(51) International Patent Classification [6] : **C07K 14/00, C12N 15/62**	**A1**	(11) International Publication Number: **WO 98/47916** (43) International Publication Date: 29 October 1998 (29.10.98)

(71) Applicant (for all designated States except US): PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 1350 Massachusetts Avenue, Cambridge, MA 02138 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): YOUNG, John [GB/US]; 388 Lexington Street, Newton, MA 02166 (US). SNITKOVSKY, Sophie [—/US]; 382 Ocean Avenue #401, Revere, MA 02151 (US).

(74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).

(54) Title: BIFUNCTIONAL POLYPEPTIDES FOR CELL-SPECIFIC VIRAL TARGETING

(57) Abstract

    The invention relates to a novel bifunctional molecule comprising a first binding moiety which binds to a surface molecule on a target cell; and a second binding moiety which binds to a surface molecule on a viral vector. The bifunctional molecule targets the viral vector to the target cell with improved infectivity and selectivity. The molecule can be used, for example, in *in vitro* and *in vivo* gene delivery methods.

BIFUNCTIONAL POLYPEPTIDES
FOR CELL-SPECIFIC VIRAL TARGETING

RELATED APPLICATION

     This application is a continuation-in-part of U.S.

5 Serial No. 08/844,359, filed April 18, 1997, the
teachings of which are incorporated herein by reference,
in their entirety.

GOVERNMENT FUNDING

     The invention described herein was supported in

10 whole or in part by Grant No. R29CA6200 from the
National Institutes of Health. The government has
certain rights in the invention.

BACKGROUND OF THE INVENTION

     Many clinical trials for gene delivery have been

15 and are presently employing viral vectors which include
in their genome a nucleic acid sequence encoding a
desired protein. Viral vectors which have been developed
for gene delivery include retroviruses, adenoviruses and
adeno-associated viruses. In recent years, significant

20 advances have occurred in the development of viral
vectors for gene delivery to increase infectivity and
decrease their ability to replicate *in vivo*, for
example.

     It has been recognized that it is desirable in some

25 instances to target the viral vector to a specific cell
type. Cell-specific targeting has been suggested for
retroviral vectors. These viruses contain two envelope
glycoprotein subunits designated surface (SU) and

-2-

transmembrane (TM) which form an oligomeric complex on the viral surface and mediate viral entry. The SU protein contains the viral receptor binding determinants whereas the TM protein contains a hydrophobic

5   transmembrane region and a separate hydrophobic segment that mediates virus-cell membrane fusion (Weiss, R.A., 1993, "Cellular receptors and viral glycoproteins involved in retrovirus entry," p. 1-107, in J.A. Levy (ed.), *The Retroviridae*, Vol. 2. Plenum press, New

10 York).

To date, there have been several reports of cell type-specific retrovirus targeting. In one case, retroviral particles were chemically modified with lactose, a procedure which resulted in specific

15 infection of human cells that expressed the asialogly-coprotein receptor (Neda, H., 1991, *J. Biol. Chem.* *266*:14143-14149). In another case, a low level of cell type-specific viral infection was achieved by forming an antibody bridge between a retroviral SU protein and

20 specific host cell surface proteins (Roux, P., 1989, *Proc. Natl. Acad. Sci. USA 86*:9079). An alternative strategy that has been employed reengineered retroviral SU proteins to contain cell type-specific ligands or single chain antibodies that bind to specific cell

25 surface proteins (Valsesia-Wittmann, S., 1994, *J. Virol.* *68*:4609-4619; Kasahara, Y., 1994, *Science 266*:1373-1376; Han, X., 1995, *Proc. Natl. Acad. Sci. USA 92*:9747-9751; Matano, T., 1995, *J. Gen. Virol. 76*:3165-3169; Valsesia-Wittmann, 1996, *J. Virol. 70*:2059-2064; Cosset, F-L.,

30 1995, *J. Virol. 69*:6314-6322; Nilson, B.H.K., 1996, *Gene Therapy 3*:280-286; Etienne-Julan, M., 1992, *J. Virol.* *69*:2659-2663; Somia, N., 1995, *Proc. Natl. Acad. Sci USA*

92:7570-7574; Russell, S.J., 1993, *Nucleic Acids Res.* 21:1081-1085; Marin, M., 1996, *J. Virol.* 70:2957-2962). This latter approach requires a) the synthesis of recombinant envelope-ligand proteins in virus producing

5 cells, b) the incorporation of the recombinant ligand-envelope fusion proteins (and in some cases also wild-type envelope proteins) onto viral surfaces, c) binding of the recombinant ligand-envelope fusion to a specific cell surface receptor, and d) fusion of the viral

10 membrane with the host cell membrane to allow infection.

The current strategies for retroviral vector targeting suffer from a number of limitations which severely restrict the utility of these vectors for a number of gene therapy approaches. These problems are

15 discussed in Cosset, F-L and Russell, S.J., 1996, *Gene Therapy* 3:946-956. These problems include the fact that it is difficult to manipulate these proteins "without impairing some critical envelope functions;" "Only a few variable peptides or loops in the SU glycoprotein have

20 been found to tolerate substitution by foreign peptides;" in some cases the need for co-expressed wild-type envelope proteins "has not been adequately explained and greatly complicates the process whereby targeted vector particles are generated;" in general

25 these strategies give rise to viruses that infect cells with a low efficiency (Cosset, 1996, *Gene Therapy* 3:946-956).

Thus, there is a continued need to develop new and improved methods for targeting viral vectors to specific

30 cell types.

-4-

SUMMARY OF THE INVENTION

This invention is based upon the discovery that bifunctional molecules, e.g., polypeptides, comprising a specific binding and entry moiety, or partner, to a viral vector and a specific binding moiety, or partner, to a target cell results in efficient binding and infection of the cell and, thereby, gene delivery. Thus the invention relates to a bifunctional molecule comprising a first binding moiety which binds to a surface protein on a target cell, a second binding moiety which binds to a surface protein on a viral vector and activates viral entry and, optionally, a linking moiety. In a preferred embodiment, the first binding polypeptide is a ligand to a cell-type specific cellular receptor and/or the second binding polypeptide is a polypeptide which has an amino acid sequence which is the same or substantially the same as an amino acid sequence of at least the viral-binding portion or fragment of a native extracellular domain of a viral cellular receptor.

In another embodiment the first binding moiety binds to a cell surface molecule on a target cell and the second binding moiety to a viral surface molecule. The target cell surface molecule can be a peptide, sugar, lipid, anion or cation, or any combination thereof which are required for specific, high affinity binding and activate viral entry into the target cell.

The invention also includes nucleic acid molecules which encode, independently or together, polypeptide binding moieties of the above bifunctional molecule. Also envisioned are host cells which comprise the nucleic acid molecules of the invention and express the

polypeptide binding moieties separately or together as a bifunctional molecule.

In yet another embodiment, the invention includes methods for preparing the bifunctional molecules of the

5   invention which includes maintaining one or more recombinant host cells which express the bifunctional molecule (e.g., which contain one or more nucleic acid molecules encoding the bifunctional molecule) under conditions suitable for expression. Alternatively, the

10   bifunctional molecules can be prepared via chemical syntheses, such as conjugating the two binding moieties.

The invention also includes methods for delivering a viral vector, such as a retroviral vector, to a cell, *in vitro* or *in vivo*, comprising contacting the cell with

15   a bifunctional molecule, as described herein. The retroviral vector typically encodes a heterologous protein.

In yet another embodiment, the invention relates to methods of screening ligand and ligand-cell specific

20   receptor binding pairs using bifunctional molecules.

The inventions which are described herein provide alternative and improved mechanisms for the successful gene delivery to specific cell types via viral vectors. In contrast to the prior art, the present invention does

25   not require the careful incorporation of a cellular binding partner into the viral particle without disturbing the conformation of a viral protein (e.g. the env protein) required for proper binding and cell entry. Neither does it require the specific cloning and

30   elucidation of the viral protein required for binding and the mechanisms for infection. The invention thereby

-6-

offers greater opportunities in cell-type specific
targeting than previously offered in the prior art.

BRIEF DESCRIPTION OF THE DRAWINGS
      Figure 1 schematically illustrates the use of the
5 bifunctional molecule in binding and inducing infection
of a cell.
      Figure 2 is a bar graph which illustrates TVA-EGF
binding to mouse L cells expressing EGF receptors
lacking kinase activity (M5) and wild type EGF receptors
10 (T23) but not to mouse L cells lacking EGF receptors
(B82).
      Figure 3 is a line graph which illustrates the EGF
receptor specificity of TVA-EGF cell surface binding to
M5 cells.
15   Figure 4 is a three dimensional scatter diagram
which illustrates differences in the time required for a
bifunctional molecule containing TVA and EGF to be
cleared from the cell surface of M5 and T23 cells.
      Figure 5 is a bar graph which illustrates the avian
20 leukosis viral (ALV) infection of M5 cells which express
the epidermal growth factor EGF receptor with a
bifunctional molecule containing TVA and EGF (TVA-EGF).
      Figure 6 is a bar graph which illustrates TVA-EGF
dependent ALV-A infection of M5 cells, which express the
25 EGF receptor, but not B82 cells, which do not express
the EGF receptor.
      Figure 7 is a bar graph which illustrates TVA-EGF
dose dependent effects on ALV-A infectivity of T23 and
M5 cells but not in B82 cells.

Figure 8 is a bar graph which illustrates the EGF
receptor specificity of TVA-EGF mediated ALV-A infection
in M5 cells.

Figure 9 schematically illustrates binding of the
5   TVA-EGF bifunctional molecule to the EGF receptor on a
target cell (EGF binding moiety) and the Env protein of
a viral vector (TVA binding moiety).

DETAILED DESCRIPTION OF THE INVENTION

As set forth above, the present invention relates
10   to the discovery that viral vectors can be targeted to
specific cell-types by contacting the viral vectors and
cells with bifunctional molecules that possess moieties
which bind to surface proteins of both the vector and
cell. The bifunctional molecule thus comprises a first
15   binding moiety which binds to a surface protein on a
target cell and a second binding moiety which binds to a
surface protein on a viral vector and induces viral
entry.

Viral vectors include retrovirus, adenovirus,
20   parvovirus (e.g., adeno-associated viruses),
coronavirus, negative strand RNA viruses such as
orthomyxovirus (e.g., influenza virus), rhabdovirus
(e.g., rabies and vesicular stomatitis virus),
paramyxovirus (e.g. measles and Sendai), positive strand
25   RNA viruses such as picornavirus and alphavirus, and
double stranded DNA viruses including adenovirus,
herpesvirus (e.g., Herpes Simplex virus types 1 and 2,
Epstein-Barr virus, cytomegalovirus), and poxvirus
(e.g., vaccinia, fowlpox and canarypox). Other viruses
30   include Norwalk virus, togavirus, flavivirus,
reoviruses, papovavirus, hepadnavirus, and hepatitis

-8-

virus, for example. Examples of retroviruses include:
avian leukosis-sarcoma, mammalian C-type, B-type
viruses, D-type viruses, HTLV-BLV group, lentivirus,
spumavirus (Coffin, J.M., 1996, Retroviridae: The
5   viruses and their replication. in Fundamental Virology,
Third Edition, edited by B.N. Fields, D.M. Knipe, P.M.
Howley, et al. Lippincott-RavenPublishers,
Philadelphia). Other examples include murine
leukemia viruses, murine sarcoma viruses, mouse mammary
10   tumor virus, bovine leukemia virus, feline leukemia
virus, feline sarcoma virus, avian leukemia virus, human
T-cell leukemia virus, baboon endogenous virus, Gibbon
ape leukemia virus, Mason Pfizer monkey virus, simian
immunodeficiency virus, simian sarcoma virus, Rous
15   sarcoma virus and lentiviruses.

        Retroviruses are a family of RNA viruses which
infect cells in a two step mechanism. The first step of
infection is the binding of the viral particle via the
surface protein of the retrovirus envelope (env) protein
20   and viral and cellular membrane fusion for viral uptake
via the transmembrane protein of the env protein. This
is discussed in more detail above. The env protein is
largely responsible for the specificity (between cell-
types and between species) of the infectivity of
25   retroviral vectors.

        Adenoviruses have a linear double-stranded DNA
genome. Adenoviruses infect cells by a two step
mechanism. First a viral surface fiber protein binds
specifically to a cell surface receptor. In the case of
30   human HeLa cells, the receptor for adenoviruses 2 and 5
is designated CAR, a member of the immunoglobulin
protein superfamily, which also serves as a cellular

-9-

receptor for coxsackie B viruses (Bergelson, 1996,
Science, *275*:1320-1323). However, other viral receptors
have been described. Following receptor binding,
adenoviruses are taken up into the cell by receptor-
5   mediated endocytosis and interaction between the viral
penton base protein and cellular integrins is necessary
for viral entry (Wickham, 1993, *Cell* 73:309; Bai, 1994,
J. Virol 68:5925; Goldman, 1995 *J. Virol.* 69:5951;
Huang, 1996, *J. Virol.* 70:4502). The viral DNA is
10   replicated in the cell extrachromosomally (Horwitz,
M.S., 1996 "Adenoviruses," in *Fields Virology*, Third
Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley
et al., Lippincott-Raven Publishers, Philadelphia, PA).

Recombinant adenoviral vectors are generated by a
15   variety of techniques that include introducing the
desired gene of interest into a bacterial plasmid at a
site flanked by adenovirus sequences. These sequences
provide control elements for gene expression and serve
as sites for recombination with a compatible adenoviral
20   genome when cotransfected together into an appropriate
mammalian cell line (Horwitz, M.S., 1996 "Adenoviruses,"
in *Fields Virology*, Third Edition edited by B.N. Fields,
D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven
Publishers, Philadelphia, PA).

25   Adeno-associated viruses (AAV) have a linear
single-stranded DNA genome and their receptor has not
yet been described. These viruses only undergo
productive infection if the infected cells are
coinfected with a helper virus (e.g., adeno- or
30   herpesvirus) otherwise the genome becomes integrated in
a latent state at a specific site on a human chromosome
(Linden, 1996, *Proc. Natl. Acad. Sci. USA 93*:11288-

11294; Berns, K.J., "Parvoviridae: The viruses and their replication" in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA). Adeno-associated

5   viral vectors are typically made by replacing viral genes with desired genes of interest or instead by simply adding the terminal AAV DNA sequences (ITRs) to these genes.

The negative strand RNA viruses infect cells by a

10   variety of different mechanisms. For example, Influenza A viruses which have a segmented RNA genome, contain a surface hemagglutinin protein which binds to cell surface sialic acid receptors and mediates viral entry in a low pH endosome following receptor-mediated

15   endocytosis (Lamb, R.A. and Krug, R.M., 1996, "Orthomyxoviridae: The viruses and their replication" *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

20   Paramyxoviruses which have a non-segmented RNA genome have two surface viral proteins, the hemagglutinin (HN) and fusion protein (F), required for viral entry which occurs at neutral pH. These viruses can utilize sialic acid receptors, or protein receptors

25   (e.g., CD46 used by measles virus), for viral entry (Lamb, R.A. and Kolakofsky, D., 1996, "Paramyxoviridae: The viruses and their replication" *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers,

30   Philadelphia, PA).

Rhabdoviruses (e.g., VSV) which have a non-segmented RNA genome, contain a surface protein (G)

which binds to specific cell surface receptors and
mediates viral entry in a low pH endosome. A specific
phospholipid appears to be one of the receptors for VSV
(Wagner, R.R. and Rose, J.K., 1996, in *Fields Virology*,
5   Third Edition edited by B.N. Fields, D.M. Knipe, P.M.
Howley *et al.*, Lippincott-Raven Publishers,
Philadelphia, PA).

A number of strategies are currently available for
engineering negative strand viral RNA genomes to express
10   heterologous genes (reviewed by Palese, P., 1996, *Proc.
Natl. Acad. Sci. USA 93*:11354-11358; Schnell, M.J.,
1996, *Proc. Natl. Acad. Sci. USA 93*:11359-11365).

The positive strand RNA viruses also infect cells
by a variety of different mechanisms. For example,
15   among the picornaviruses, different members of the
immunoglobulin protein superfamily are used as cellular
receptors by poliovirus, by the major subgroups of
rhinoviruses, and by coxsackie B viruses, whereas an
integrin protein is used by some types of ecoviruses and
20   a low density lipoprotein receptor is used by minor
subgroups of rhinoviruses (Bergelson, 1996, *Science
275*:1320-1323; Rueckert, R.R., 1996, "Picornaviridae:
The viruses and their replication" *Fields Virology*,
Third Edition edited by B.N. Fields, D.M. Knipe, P.M.
25   Howley *et al.*, Lippincott-Raven Publishers,
Philadelphia, PA). Following receptor-binding, it is
not yet known precisely what role receptor-mediated
endocytosis plays for picornaviral entry, if indeed it
is required.

30   Because the picornaviruses lack a surface lipid
bilayer, their entry pathway does not involve fusion of
a viral membrane with a host cell membrane. In

-12-

contrast, the alphaviruses (e.g., Sindbis virus and
Semliki virus) do contain a surface lipid bilayer.
These viruses contain two (E1 and E2) surface proteins,
and in some cases a third (E3) surface protein important

5   for viral entry. These viruses use various cell surface
receptors. For example, Sindbis virus can use a laminin
receptor or other receptors and generally enter cells by
a pH-dependent mechanism, following receptor-mediated
endocytosis (Frolov, 1996, *Proc. Natl. Acad. Sci. USA*

10   *93*:11371-11377; Schlesinger, S. and Schlesinger, M.J.,
1996, "Togaviridae: The viruses and their replication,"
in *Fields Virology*, Third Edition edited by B.N. Fields,
D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven
Publishers, Philadelphia, PA).

15   The herpesviruses which have large double-stranded
DNA genomes, contain a number of surface glycoproteins
involved in viral entry and utilize various cell surface
receptors. For example, herpes simplex virus and
cytomegalovirus entry involves binding to a heparin

20   sulfate cell surface receptor and herpes simplex viruses
use other proteins (e.g., HVEM) for viral entry
(Montgomery, R., 1996, *Cell 87*:427-436). In contrast,
Epstein-Barr virus entry is initiated by binding to a
completely distinct cell surface receptor, CR2 (Wolf,

25   1993, *Intervirology 35*:26-39). Strategies have been
described that allow one to engineer herpes simplex
viruses, cytomegaloviruses and Epstein-Barr viruses as
vectors for heterologous gene expression (Roizman, 1996,
Proc. Natl. Acad. Sci. USA 93:11307-11312; Andreansky,

30   1996, *Proc. Natl. Acad. Sci. USA 93*:11313-11318;
Marconi, 1996, *Proc. Natl. Acad. Sci. USA 93*:11319-
11320; Mocarski, 1996, *Proc. Natl. Acad. Sci. USA*

-13-

93:11321-11326; Robertson, 1996, *Proc. Natl. Acad. Sci. USA 93*:11334-11340; Duboise, 1996, *Proc. Natl. Acad. Sci. USA 93*:11389-11394).

5   Poxviruses have large double stranded DNA genomes and enter cells by a pH-independent mechanism via receptors that remain to be defined (Moss, B., 1996: "Poxviridae: The viruses and their replication," in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven

10   Publishers, Philadelphia, PA). Poxvirus vectors have been used extensively for the expression of heterologous recombinant genes and as vaccines (Moss, B., 1996, *Proc. Natl. Acad. Sci. USA 93*:11341-11348; Paoletti, 1996, *Proc. Natl. Acad. Sci. USA 93*:11349-11353).

15   Viral vectors which have been "pseudotyped" incorporate the env protein from a retrovirus of a desired specificity into the vector. Thus, a pseudotyped virus has the env protein from a first retrovirus and core or structural proteins from a second

20   virus (e.g. a second retrovirus, an orthomyxovirus or a rhabdovirus). Viral pseudotypes have been described, for example, in Le Guen, 1992, *Proc. Natl. Acad. Sci. USA 89*:363-367; Rizvi, 1992, *Journal of Medical Primatology 21*:69-73; Takeuchi, 1992, *Virology 186*:792-

25   294; Vile *et al.*, 1991, *Virology 180*:420; Miller *et al.*, 1991, *J. Virol. 65*:2220; Landau, *et al.,,* 1991, *J. Virol. 65*:162; Emi, *et al.*, 1991, *J. Virol. 65*:1207; and Dong, *et al.*, 1992, *J. Virol 66*:7374. The present invention can thus target a pseudotyped viral vector

30   with a bifunctional molecule which binds to the target cell surface protein and binds to the heterologous env protein, inducing viral entry.

-14-

Typically, retroviral vectors are manufactured by "packaged cell lines" which provide the retroviral proteins necessary for infection (e.g., env, gag and pol), but are incapable of replication upon infection.

5   See, for example, Miller, AD, 1992, Current Topics in Microbiology and Immunology, Vol. 158, pages 1-24.

The viral vectors employed in the present invention can be used for polynucleotide or gene delivery to a cell or animal. The polynucleotide to be delivered to

10   the cell or animal can include a polynucleotide native to the viral vector or heterologous to the vector. In a preferred embodiment, the viral vector has been engineered to contain a polynucleotide which is itself therapeutic or encodes a therapeutic protein, which will

15   be discussed in more detail below.

The invention, as provided for herein, is a mechanism by which the viral vector can more efficiently deliver a desired gene to a cell or animal by indirectly binding the vector to the target cell via a bifunctional

20   molecule comprising a first and second binding moiety.

A "binding moiety," as that term is defined herein, includes a chemical entity which binds to the specified target molecule. The binding can be via a covalent bond, ionic bonding, hydrogen bonding or other

25   mechanism. The binding moiety can be a peptide (including post-translationally modified proteins, such as amidated, demethylated, glycosylated or phosphorylated proteins), sugar, lipid, steroid, nucleic acid, small molecule, anion or cation, or combination thereof

30   which binds the specified target molecule. Preferably, the binding will possess a high affinity. Examples of

high affinity, as that term is used herein, have an
equilibrium constant of 10^{-6} (preferably 10^{-8}) or lower.

As set forth above, the first binding moiety binds
a surface protein, or target molecule, on the target
5   cell. The "target cell" is defined as the cell which is
intended to be infected by the viral vector. Typically,
the target cell is of animal origin and can be a stem
cell or somatic cells. Suitable animal cells for use on
the claimed invention can be of, for example, mammalian
10 and avian origin. Examples of mammalian cells include
human, bovine, ovine, porcine, murine, rabbit cells.
The cell may be an embryonic cell, bone marrow stem cell
or other progenitor cell. Where the cell is a somatic
cell, the cell can be, for example, an epithelial cell,
15 fibroblast, smooth muscle cell, blood cell (including a
hematopoietic cell, red blood cell, T-cell, B-cell,
etc.), tumor cell, cardiac muscle cell, macrophage,
dendritic cell, neuronal cell (e.g., a glial cell or
astrocyte), or pathogen-infected cell (e.g., those
20 infected by bacteria, viruses, virusoids, parasites, or
prions).

Typically, cells isolated from a specific tissue
(such as epithelium, fibroblast or hematopoietic cells)
are categorized as a "cell-type." The cells can be
25 obtained commercially or from a depository or obtained
directly from an animal, such as by biopsy.
Alternatively, the cell need not be isolated at all from
the animal where, for example, it is desirable to
deliver the vector to the animal in gene therapy.
30 Cells are typically characterized by markers
expressed at their surface that are termed "surface
markers". These surface markers include surface

-16-

proteins or target molecules, such as cellular
receptors, adhesion molecules, transporter proteins,
components of the extracellular matrix and the like.
These markers, proteins and molecules also include
5   specific carbohydrates and/or lipid moieties, for
example, conjugated to proteins. The first binding
moiety binds to one or more surface proteins on the
target cell. Surface proteins can be tissue- or cell-
type specific (e.g. as in surface markers) or can be
10   found on the surface of many cells. Typically, the
surface marker, protein or molecule is a transmembrane
protein with one or more domains which extend to the
exterior of the cell (e.g. the extracellular domain).
Where cell-type specific delivery is desired (as in *in*
15   *vivo* delivery of a viral vector), the surface protein
selected for the invention is preferably specific to the
tissue. By "specific" to the tissue, it is meant that
the protein be present on the targeted cell-type but not
present (or present at a significantly lower concentra-
20   tion) on a substantial number of other cell-types.
While it can be desirable, and even preferred, to select
a surface protein which is unique to the target cell, it
is not required for the claimed invention. It is to be
appreciated, however, that specific delivery may not be
25   required where the cell or cells are contacted with the
viral vector in pure or substantially pure form, such as
can be the case in an *in vitro* gene transfer. As such,
the surface protein or targeted protein for the first
binding moiety may be present on many different cell-
30   types, specific or even unique to the targeted cell-
type.

        As set forth above, the surface protein can be a
cellular receptor or other protein, preferably a
cellular receptor. Examples of cellular receptors
include receptors for cytokines, growth factors, and
5   include, in particular epidermal growth factor
receptors, platelet derived growth factor receptors,
interferon receptors, insulin receptors, proteins with
seven transmembrane domains including chemokine
receptors and frizzled related proteins (Wnt receptors),
10 immunoglobulin-related proteins including MHC proteins,
CD4, CD8, ICAM-1, etc., tumor necrosis factor-related
proteins including the type I and type II TNF receptors,
Fas, DR3, DR4, CAR1, etc., low density lipoprotein
receptor, integrins, and, in some instances, the Fc
15 receptor.

        Other examples of surface proteins which can be
used in the present invention include cell-bound tumor
antigens. Many of these surface proteins are
commercially available and/or have been characterized in
20 the art, including the amino acid and nucleic acid
sequences, which can be obtained from, for example,
GENBANK, as well as the specific binding characteristics
and domains. Cytokine and chemokine receptors are
reviewed for example, in Miyama, *et al. Ann. Rev.*
25 *Immunol.*, *10*:295-331 (1992), Murphy, *Ann. Rev. Immunol.*
12:593-633 (1994) and Miller *et al. Critical Reviews in*
Immunol. 12:17-46 (1992).

        Typically, the first binding moiety is selected or
derived from native ligands or binding partners to the
30 surface protein of the target cell. In the case of a
cellular receptor, for example, for a cytokine or growth
factor, the first binding moiety can be a polypeptide

comprising at least the receptor-binding portion of the
native ligand. A "native ligand" or "native binding
partner" is defined herein as the molecule naturally
produced by, for example, the animal or species which
5   binds to the surface protein in nature. Preferably, the
first binding moiety is a polypeptide or protein. As
such, the native ligand of a cytokine receptor is the
native cytokine. In another embodiment, the first
binding moiety can comprise a binding fragment of an
10   antibody, such as the variable region or a single chain
antibody.

Preferably, the first binding moiety is a poly-
peptide ligand to a cellular receptor. Examples of
preferred ligands are growth factors, epidermal growth
15   factor, interleukins, GM-CSF, G-CSF, M-CSF, EPO, TNF,
interferons, and chemokines. Generally, the cellular
receptor is not presented on the cell which expresses
the viral receptor (e.g., a cell receptor which is bound
by the virus during infection). In one embodiment, the
20   cellular receptor which binds to the first binding
moiety is not an Fc receptor. Thus, in this embodiment,
the bifunctional molecule is not an immunoadhesin.

The first binding moiety can have an amino acid
sequence which is the same or substantially the same as
25   an amino acid sequence of at least the receptor-binding
portion of a native ligand for the cellular receptor.
Similar to cellular receptors, many of the corresponding
ligands have been identified, sequenced and character-
ized, including the portions thereof which bind to the
30   receptor. The first binding moiety can, therefore,
include the same or substantially the same sequence of
the entire native ligand. Alternatively, first binding

-19-

moiety comprises the receptor binding portion of the
native ligand, eliminating, in some cases, the effector
function of the ligand.

In another embodiment, the first binding moiety is
5   selected or derived from native ligands or binding
partners to a cellular surface molecule of the target
cell. A "cellular surface molecule" as defined herein
can be a peptide (including post-translationally
modified proteins, such as amidated, demethylated,
10   methylated, prenylated, palmitoylated, glycosylated,
myristylated, acetylated or phosphorylated proteins),
sugar, lipid, steriod, anion or cation, or a combination
thereof which binds the first binding moiety.
Preferably, the binding of the cellular surface molecule
15   to the binding moiety of the bifunctional molecule will
be of high affinity. Examples of high affinity have an
equilibrum constant of 10^{-6} (preferably 10^{-8}) or lower.

The cellular surface molecule need not be
"specific" for the target cell. However, the cellular
20   surface molecule is specific for the desired viral
vector. For example, specific delivery of Influenza A
viral vectors can employ sialic acid cellular surface
molecules for entry into a target cell whereas targeting
of VSV viral vectors can employ a phospholipid as the
25   surface molecule. As such, the cellular surface
molecule for the first binding moiety can be present on
many different cell-types, specific or even unique to
the target cell.

In other embodiments, the effector function can be
30   desirable, thereby stimulating or modulating the
cellular activity of the target cell which can enhance
therapy. An example of where such a therapy can be

-20-

desirable is in the delivery of a negative selection
marker or suicide protein to a tumor where the target
cell is a lymphokine and the ligand is a cytokine.
Where the lymphokine is stimulated, the cell, in

5   addition to delivering the viral vector, can also
possess therapeutic value in the recruitment of an
endogenous immune response against the tumor, thereby
increasing the therapeutic benefit of the therapy.

The phrase "substantially the same sequence" is

10   intended to include sequences which bind the surface
protein and possess a high percentage of (e.g., at least
about 90%, preferably at least about 95%) sequence
identity with the native sequence. The modifications to
the sequence can be conserved or non-conserved, natural

15   and unnatural, amino acids and are preferably outside of
the binding domain. Amino acids of the native sequence
for substitution, deletion, or conservation can be
identified, for example, by a sequence alignment between
proteins from related species or other related proteins.

20   Where the first binding moiety comprises a binding
fragment of an antibody, many antibodies to surface
proteins are known or are commercially available, as are
the amino acid sequences which are responsible for
binding. Alternatively, novel antibodies can be

25   prepared by methods known in the art, such as by Harlow
and Lane, "Antibodies, A Laboratory Manual," Cold Spring
Harbor Laboratory (1988).

Similar to the first binding moiety, the second
binding moiety is a chemical entity which binds to the

30   specified viral target molecule on the viral vector,
generally a surface protein of the viral particle. The
binding can be via a covalent bond, ionic bonding,

-21-

hydrogen bonding or other mechanism. The binding moiety
can be a peptide, sugar, lipid, steroid, nucleic acid,
small molecule, anion or cation, or combination thereof
which binds the specified viral target molecule.

5   Generally, the second binding moiety of the bifunctional
molecule is also a polypeptide. One embodiment of the
second binding moiety comprises an antigen-binding
fragment of an antibody which recognizes and binds to
the viral vector. However, where one binding moiety

10   comprises an antibody fragment, it is preferred that the
other binding molecule does not comprise an antibody
fragment.

In a more preferred embodiment, the second binding
moiety is a polypeptide which has an amino acid sequence

15   which is the same or substantially the same as an amino
acid sequence of at least the viral-binding portion of a
native extracellular domain of a viral cellular
receptor, such as the viral cellular receptor is on a
cell which is subject to infection by the viral vector.

20   The viral surface protein is thus generally the viral
protein which binds to this cell and activates or
otherwise induces steps for viral entry and, thus,
infection. Where the viral vector is a pseudotyped
viral vector (such as where the vector is a retrovirus

25   containing the envelope protein of another retrovirus,
of an orthomyxovirus or of a rhabdovirus), as discussed
above, it is understood that the viral cellular receptor
generally corresponds to the env protein incorporated
into the viral particle. In the case where the viral

30   surface protein is an env protein, the second binding
partner generally binds the env SU protein and, thereby,
activates the env TM protein, inducing or mediating

-22-

virus-cell membrane fusion and viral entry. As such, a
"viral cellular receptor" is a receptor molecule in or
on a cell which binds to a natural or non-natural viral
particle or protein, for example, in the course of
5   infection. Non-natural viral particles include
pseudotyped viruses, replication defective viruses,
attenuated viruses, or recombinantly engineered viruses.

Examples of viral cellular receptors include the
subgroup A avian leukosis virus receptor, the subgroup B
10   and D avian leukosis virus receptor, the CD4 receptor
for human and simian immunodeficiency viruses, the
amphotropic and ecotropic murine leukemia virus
receptors, the bovine leukemia virus receptor, the
gibbon ape leukemia virus receptor, the poliovirus
15   receptor, the major subgroup rhinovirus receptor (ICAM-
1), the adenovirus and coxsackie virus receptor (CAR),
the coronavirus receptors (e.g. aminopeptidase N and
MHVR), and sialic acid and other carbohydrate receptors
(e.g., for orthomyxoviruses and paramyxoviruses). Many
20   viral cellular receptors are known in the art, including
the sequences thereof.

Cellular receptors are generally transmembrane
proteins comprising intracellular, transmembrane
(characterized by highly hydrophobic regions in the
25   sequence) and extracellular domains. It is generally
preferred that the second binding moiety comprises the
native extracellular domain of the receptor molecule.
As above with the first binding moiety, the second
binding moiety can alternatively comprise the viral
30   binding moiety of the extracellular domain of the
receptor and mutations of the extracellular domain. The
viral binding moiety can be identified or confirmed by

manufacturing various fragments of the extracellular
domain and screening them for their ability to bind. As
above, viral-binding mutations to the extracellular
domain can be manufactured as well. Mutations to the
5   extracellular domain include conservative and non-
conservative amino acid substitutions, particularly
those not implicated in the binding of the viral
particle. The phrase "substantially the same sequence"
is intended to include sequences which bind the viral
10 protein and possess a high percentage of (e.g., at least
about 90%, preferably at least about 95%) amino acid
sequence identity with the native sequence. The
modifications to the sequence can be conserved or non-
conserved amino acids.
15   The phrase "conservative amino acid substitutions"
is intended to mean amino acids which possess similar
side chains (e.g., hydrophobic, hydrophilic, aromatic,
etc.), as is known in the art.
        The first and second binding moieties can be
20 directly bonded together or through a linking moiety.
Where one or both of the moieties are polypeptides, a
peptide bond or peptide linker is preferred, thereby
obtaining a "fusion protein" of the two moieties which
can be expressed by a single nucleic acid construct in
25 series. The two moieties can alternatively be linked
directly or indirectly other than via a peptide bond or
peptide linker, thereby obtaining a "conjugate".
        Where the moieties are directly bonded to each
other, the bond can be covalent, as in a peptide bond,
30 ionic bonding or hydrogen bonding. Where the bond is a
peptide bond, the first moiety can be bonded to the N-
terminus of the second moiety via the C-terminus, or

-24-

vice versa.   It is acknowledged that one fusion protein
may possess greater activity than a second fusion
protein due to conformational or steric considerations.

Where one or more of the moieties are not poly-
5   peptides, they can be joined via chemical reaction
through functional groups present on each moiety which,
under the appropriate conditions, will react with each
other.   For example, acid groups (or activated
derivatives thereof) can be reacted with amines,
10   alcohols or thiols to form amide or ester bonds, as is
known in the art.

Alternatively and advantageously, a linking moiety
is employed to link the two moieties.   The linker can
preferably be a flexible linker and sufficient in length
15   to separate the moieties in space, thereby not
restricting the ability of the bifunctional molecule to
bind independently and maintain the proper conformation.
Again, where both moieties are polypeptides, the linker
moiety will generally be a peptide, polypeptide, or a
20   "pseudopeptide".   A "pseudopeptide" is a bifunctional
linker which contains at least one non-amino acid and
reacts to form a peptide bond, or other bond, with the
terminal amine or carboxyl group of the moiety.   For
example, a peptide characterized by substitution of the
25   terminal amine for a carboxyl group can function to
react with the amine terminus of each moiety.   Such as
linker is considered to be a "pseudopeptide." Similarly,
a peptide characterized by substitution of the terminal
carboxyl for an amine group can function to react with
30   the carboxyl terminus of each moiety.

Generally, however, the linker will be a peptide
linker which will link the amine terminus of one moiety

-25-

to the carboxyl terminus of the second moiety. One
advantage to such a molecule is the ability to express
the bifunctional molecule as a fusion protein in a
recombinant host cell with a single nucleic acid
5   construct.

Preferred peptide linkers can be obtained from
immunoglobulin hinge regions, such as a proline rich
region. Also, linkers can be characterized by little
steric hindrance, thereby permitting maximal independent
10   movement of the two moieties, such as with a polyglycine
linker. Alternatively, the linker selected to be
reactive to or inert to cellular proteases can be
desirable. In another embodiment, the linker can be
selected to avoid or minimize an immune response against
15   the fusion protein. The length of the linker also is
not particularly critical. Typically, the length of the
linker will be between about 2 and about 20 amino acids.
As can be seen, the selection of the particular linking
group is not critical to the invention.

20   In yet another embodiment, the linker can be a
bifunctional compound which will react with other
functional groups on the two binding moieties, such as
in the reaction of acids and amines or alcohols (as
present in peptides, carbohydrates and lipids, for
25   example) in the formation of amides or esters.

A preferred combination of the above first and
second binding moieties includes the selection of a
polypeptide ligand to a cell-type specific cellular
receptor linked, via a peptide linker through a terminus
30   of the ligand to the terminus of at least the viral-
binding moiety of the extracellular domain of a viral

-26-

cellular receptor (or a mutant thereof, as defined above).

For example, the C-terminus of the first binding polypeptide is linked to the N-terminus of the second
5   binding polypeptide via the polypeptide linker or the N-terminus of the first binding polypeptide is linked to the C-terminus of the second binding polypeptide via the polypeptide linker.

A particularly preferred combination of these
10   elements is for targeting a retroviral vector to a cell-type comprises a receptor-binding fragment of a polypeptide ligand which binds a cellular receptor specific to the desired cell-type and a viral-binding fragment of a cellular receptor for the retroviral
15   vector. For example, the polypeptide ligand can be human epidermal growth factor. Where the retroviral vector is avian leukosis virus, the cellular receptor can be Tva. The two polypeptides can then be linked via a hinge region, such as a proline-rich hinge region.

20   In another aspect of the invention, peptidomimetics (molecules which are not polypeptides, but which mimic aspects of their structures to bind to the same site), that are based upon the above-described polypeptides, can also be used as binding moieties of the bifunctional
25   molecules. For example, polysaccharides can be prepared that have the same functional groups as the polypeptide binding moieties of the invention, and which interact with target cell and viral surface molecules in a similar manner. Peptidomimetics can be designed, for
30   example, by establishing the three dimensional structure of the polypeptide in the environment in which it is bound or will bind to the target cell or viral vector.

-27-

The peptidomimetic comprises at least two components, a
binding entity or entities and a backbone or supporting
structure entity.

The binding entities of the peptidomimetic are the
5   chemical atoms or groups which will react or complex (as
in the formation of a hydrogen or covalent bond) with
the target cell or viral surface molecule. In general,
the binding entities in a peptidomimetic are the same as
the polypeptide of the bifunctional molecule.
10   Alternatively, the binding entities can be an atom or
chemical group which will react with the target cell or
viral surface molecule in the same or similar manner as
the polypeptide. Examples of binding entities suitable
for use in designing a peptidomimetic for a basic amino
15   acid in a polypeptide are nitrogen containing groups,
such as amines, ammoniums, guanidines and amides or
phosphoniums. Examples of binding entities suitable for
use in designing a peptidomimetic for an acidic amino
acid in a polypeptide can be, for example, carboxyl,
20   lower alkyl carboxylic acid ester, sulfonic acid, a
lower alkyl sulfonic acid ester or a phosphorous acid or
ester thereof.

The supporting structure is the chemical entity
that, when bound to the binding moiety or moieties,
25   provides the three dimensional configuration of the
peptidomimetic. The supporting structure can be organic
or inorganic. Examples of organic supporting structures
include polysaccharides, polymers (such as, polyvinyl
alcohol or polylactide). It is preferred that the
30   supporting structure possess substantially the same size
and dimensions as the polypeptide backbone or supporting
structure. This can be determined by calculating or

-28-

measuring the size of the atoms and bonds of the
polypeptide and peptidomimetic. For example, the
nitrogen of the peptide bond can be substituted with
oxygen or sulfur, thereby forming a polyester backbone.
5   Likewise, the carbonyl of the peptide bond can be
substituted with a sulfonyl group or sulfonyl group,
thereby forming a polyamide. Reverse amides of the
peptide can be made (e.g., substituting one or more
-CONH- groups for a -NHCO- group). In addition, the
10 peptide backbone can be substituted with a polysilane
backbone.

    These peptidomimetic compounds can be manufactured
by art-known and art-recognized methods. For example, a
polyester corresponding to the peptide RRKRK can be
15 prepared by the substituting a hydroxyl group for each
corresponding amine group on the R and K amino acids,
thereby preparing a hydroxyacid and sequentially
esterifying the hydroxyacids, optionally blocking the
basic side chains and acids to minimize side reactions.
20 Determining an appropriate chemical synthesis route can
generally be readily identified upon determining the
chemical structure using no more than routine skill.

    Also encompassed by the present invention are the
use of bifunctional molecules for detecting ligand and
25 ligand-cell specific receptor binding pairs. The ligand
and/or receptor can be peptides (including post-
translationally modified proteins) and/or small
molecules (including sugars, steroids, lipids, anions or
cations). The ligands and ligand-cell specific
30 receptors can be known or unknown. Where the ligand is
known and the receptor is unknown, ligand-cell specific
receptors can be identified, for example, by screening

-29-

host cells transfected with nucleotides encoding
potential receptors. For example, the ligands can be
secreted (such as chemokines) or non-secreted (such as
the extracellular domains of chemokines receptors)
5   proteins.

A library of host cells displaying putative ligand-
cell surface receptors can be obtained by transfecting
suitable host cells with nucleic acid constructs,
including but not limited to cDNA or genomic libraries,
10 under appropriate regulatory control to result in the
expression of cell-surface receptors on the host cell.
The bifunctional molecule with ligand and an appropriate
viral vector, e.g., containing an inducible reporter
gene (such as β-galactosidase or chloramphenicol acetyl
15. transferase), is added to the population of host cells
under conditions suitable for viral infection.
Following infection, infection can be detected. For
example, reporter gene expression can be induced under
appropriate conditions and the host cells which express
20 the ligand receptor can be identified. Experimental
conditions for viral vector expression and induction and
detection of reporter genes are well known in the art.
A similar approach can be used to select unknown ligands
in the case where the ligand-cell specific receptors are
25 known and the ligand is unknown. In this embodiment, a
library of bifunctional molecules with putative ligands
(e.g., chemokines) can be obtained and contacted with
one or more host cells displaying cell surface receptors
and an appropriate viral vector. Infection can be
30 detected as described above.

Thus, the invention relates to a method for
detecting ligand-receptor binding pairs comprising

-30-

contacting, under conditions suitable for infection, a
mixture comprising a viral vector, a bifunctional
molecule and a host cell, wherein the bifunctional
molecule comprises a ligand and a binding moiety which
5   binds to a viral vector surface molecule on the viral
vector and activates viral entry into a host cell
expressing a receptor. Ligand binding to a receptor can
be indicated by viral vector infection of the host cell
by, for example, the use of reporter genes. The host
10   cells can display receptors distinct for the known
ligand or can express recombinant receptors comprising a
collection of nucleic acid constructs encoding ligand-
cell surface receptors.

A similar approach can be used to identify unknown
15   ligands wherein the cell surface receptor is known,
where the bifunctional molecule comprises a receptor and
a binding moiety which binds a viral surface molecule.
The host cell expresses a distinct ligand or a
collection of recombinant ligands. Ligand-receptor
20   binding can be detected following infection of the viral
vector to the host cell.

The bifunctional molecules can be manufactured
according to methods generally known in the art. For
example, where one or both of the binding moieties is a
25   nonpeptide the bifunctional molecule can be manufactured
employing known organic synthesis methods useful for
reacting a functional or reactive group on the moiety
with a functional or reactive group on the other moiety
or, preferably, a linker. In carrying out the
30   synthesis, derivation or inactivation of the functional
group(s) required for binding to the moiety's binding
partner should be avoided. Appropriate syntheses are

-31-

highly dependent upon the chemical nature of the binding moiety and, generally, can be selected from an advanced organic chemistry text, such as March, *et al.* *Advanced Organic Chemistry*, 3rd Edition (1985) John E. Wiley &

5   Sons, Inc., New York, NY, or other known methods.

Where the binding moieties are polypeptides, the bifunctional molecule can be a conjugate or a fusion protein and manufactured according to known methods. Where a fusion protein is desired, the molecule can be

10 manufactured according to known methods of recombinant DNA technology. For example, the fusion protein can be expressed by a nucleic acid molecule comprising sequences which code for both moieties, such as by a fusion gene. Thus, the invention further relates to

15 nucleic acid molecules which encode the bifunctional molecule.

The nucleic acid molecule can be double stranded or single stranded and can be a DNA molecule, such as cDNA or genomic DNA, or an RNA molecule. As such, the

20 nucleic acid molecule can include one or more exons, with or without, as appropriate, introns. In one example, the nucleic acid molecule contains a single open reading frame which encodes both the first binding moiety, second binding moiety and optionally a signal

25 sequence and/or a polypeptide linker, when present. By way of example in a multi-exon construct, the nucleic acid molecule contains a first exon which begins with an ATG, encodes one of the binding partners, and optionally the polypeptide linker, and ends with a splice donor

30 site. The construct further would contain an intron followed by a second exon which begins with a splice acceptor site and, optionally a polypeptide linker,

coding sequences for the other binding partner and
ending with a stop codon. Of course, alternative
combinations of these elements would be apparent to the
person of skill in the art.

5   As such, the nucleic acid molecule can include
sequences which encode the first and second binding
partners, as well as one or more of the following
optional sequences, in a functional relationship,
regulatory sequences (as will be discussed in more
10   detail below) a start codon, a signal or leader
sequence, splice donor sites, splice acceptor sites,
introns, a stop codon, transcription termination
sequences, 5' and 3' untranslated regions, polyadenyl-
ation sequences, negative and/or positive selective
15   markers, and replication sequences.

The coding regions of the nucleic acid molecule
code for the first and second binding moieties of the
bifunctional molecule and any polypeptide linkers
present. Where the binding moiety is a native ligand or
20   cellular surface protein (e.g. a cellular receptor), or
a binding fragment thereof, the nucleic acid molecule
coding regions can correspond to the native sequences
which encode the binding moiety. Because many amino
acids are encoded by a plurality of codons, the coding
25   sequence can be mutated to result in the same amino acid
sequence. This may be advantageous where a codon is
preferred by the selected host cell. Where the binding
moiety is a mutation or variant of a native sequence, as
provided above, generally, the nucleic acid sequence
30   will be mutated correspondingly. It is preferred for
ease of manufacture of the nucleic acid

sequence to maintain as much of the native sequence as possible. In one embodiment, the nucleic acid molecule shares at least about 65% sequence identity with the corresponding native sequence, preferably, at least

5 about 75% sequence. In a more preferred embodiment, the percent sequence identity is at least about 85%, and still more preferably, at least about 95%.

Recombinant nucleic acid molecules meeting these criteria comprise nucleic acids having sequences

10 identical to sequences of naturally occurring genes, including polymorphic or allelic variants, and portions (fragments) thereof, or variants of the naturally occurring genes. Such variants include mutants differing by the addition, deletion or substitution of

15 one or more residues, modified nucleic acids in which one or more residues are modified (e.g., DNA or RNA analogs), and mutants comprising one or more modified residues.

Many nucleic acid molecules coding for suitable

20 binding moieties are known in the art and can be obtained from, for example, GENBANK. Alternatively, other sequences can be employed, such as homologs of known genes.

Such homologous nucleic acids, including DNA or

25 RNA, can be detected and/or isolated by hybridization (e.g., under high stringency conditions or moderate stringency conditions). "Stringency conditions" for hybridization is a term of art which refers to the conditions of temperature and buffer concentration which

30 permit hybridization of a particular nucleic acid to a second nucleic acid in which the first nucleic acid may be perfectly complementary to the second, or the first

and second may share some degree of complementarity
which is less than perfect. For example, certain high
stringency conditions can be used which distinguish
perfectly complementary nucleic acids from those of less

5   complementarity. "High stringency conditions" and
"moderate stringency conditions" for nucleic acid
hybridizations are explained on pages 2.10.1-2.10.16
(see particularly 2.10.8-11) and pages 6.3.1-6 in
Current Protocols in Molecular Biology (Ausubel, F.M. *et*

10 *al.*, eds., Vol. 1, containing supplements up through
Supplement 29, 1995), the teachings of which are hereby
incorporated by reference. The exact conditions which
determine the stringency of hybridization depend not
only on ionic strength, temperature and the

15 concentration of destabilizing agents such as formamide,
but also on factors such as the length of the nucleic
acid sequence, base composition, percent mismatch
between hybridizing sequences and the frequency of
occurrence of subsets of that sequence within other non-

20 identical sequences. Thus, high or moderate stringency
conditions can be determined empirically.

By varying hybridization conditions from a level of
stringency at which no hybridization occurs to a level
at which hybridization is first observed, conditions

25 which will allow a given sequence to hybridize (e.g.
selectively) with the most similar sequences in the
sample can be determined.

Exemplary conditions are described in Krause, M.H.
and S.A. Aaronson, *Methods in Enzymology, 200*:546-556

30 (1991). Also, see especially page 2.10.11 in *Current
Protocols in Molecular Biology (supra)*, which describes
how to determine washing conditions for moderate or low

-35-

stringency conditions. Washing is the step in which
conditions are usually set so as to determine a minimum
level of complementarily of the hybrids. Generally,
starting from the lowest temperature at which only
5   homologous hybridization occurs, each °C by which the
final wash temperature is reduced (holding SSC
concentration constant) allows an increase by 1% in the
maximum extent of mismatching among the sequences that
hybridize. Generally, doubling the concentration of SSC
10   results in an increase in T_m of ~17°C. Using these
guidelines, the washing temperature can be determined
empirically for high, moderate or low stringency,
depending on the level of mismatch sought.

The nucleic acid molecule also preferably comprises
15   regulatory sequences. Regulatory sequences include all
cis-acting elements that control transcription and
regulation such as, promoter sequences, enhancers,
ribosomal binding sites, and transcription binding
sites. Selection of the promoter will generally depend
20   upon the desired route for expressing the protein. For
example, where the protein will be transformed into a
cell by a viral vector, preferred promoter sequences
include viral, such as retroviral or adenoviral
promoters. Examples of suitable promoters include the
25   cytomegalovirus immediate-early promoter, the retroviral
LTR, SV40, and TK promoter. Where the protein is to be
expressed in a recombinant eukaryotic or prokaryotic
cell, the selected promoter is recognized by the host
cell. A suitable promoter which can be used can include
30   the native promoter for the binding moiety which appears
first in the construct.

The elements which comprise the nucleic acid molecule can be isolated from nature, modified from native sequences or manufactured *de novo*, as described, for example, in the above-referenced texts. The
5   elements can then be isolated and fused together by methods known in the art, such as exploiting and manufacturing compatible cloning or restriction sites.

The nucleic acid molecules can be inserted into a construct which can, optionally, replicate and/or
10   integrate into a recombinant host cell, by known methods. The host cell can be a eukaryotic or prokaryotic cell and includes, for example, baculo- viruses, pichia expression systems, yeast (such as Saccharomyces), bacteria (such as, Escherichia or
15   Bacillus), animal cells or tissue, including insect or mammalian cells (such as, somatic or embryonic human cells, Chinese hamster ovary cells, HeLa cells, human 293 cells and monkey COS-7 cells, etc.).

The nucleic acid molecule can be incorporated or
20   inserted into the host cell, also, by known methods. Examples of suitable methods of transfecting or transforming cells include calcium phosphate precipita- tion, electroporation, microinjection, infection, lipofection and direct uptake. Methods for preparing
25   such recombinant host cells are described in more detail in Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual," Second Edition (1989) and Ausubel, *et al.* "Current Protocols in Molecular Biology," (1992), for example.

30   The host cell is then maintained under suitable conditions for expression and recovering the bifunctional molecule. Generally, the cells are

-37-

maintained in a suitable buffer and/or growth medium or
nutrient source for growth of the cells and expression
of the gene product(s). The growth media are not
critical to the invention, are generally known in the
5   art and include sources of carbon, nitrogen and sulfur.
Examples include Dulbeccos modified eagles media (DMEM),
RPMI-1640, M199 and Grace's insect media. Again, the
selection of a buffer is not critical to the invention.
The pH which can be selected is generally one tolerated
10   by or optimal for growth for the host cell.

The cell is maintained under a suitable temperature
and atmosphere. Anaerobic host cells are generally
maintained under anaerobic conditions. Alternatively,
the host cell is aerobic and the host cell is maintained
15   under atmospheric conditions or other suitable
conditions for growth. The temperature should also be
selected so that the host cell tolerates the process and
can be for example, between about 35° and 40°C.

The bifunctional molecule produced by the processes
20   described herein can be isolated and purified by known
means. Examples of suitable purification and isolation
processes are generally known and include ammonium
sulfate precipitation, dialysis, gel filtration, immuno-
affinity, chromatography, electrophoresis, ultrafiltra-
25   tion, microfiltration or diafiltration.

The bifunctional molecule is preferably purified
substantially prior to use, particularly where the
protein will be employed as an *in vivo* therapeutic,
although the degree of purity is not necessarily
30   critical where the molecule is to be used *in vitro*. In
one embodiment, the bifunctional molecule can be
isolated to about 50% purity (by weight), more

-38-

preferably to about 80% by weight or about 95% by
weight. It is most preferred to employ a molecule which
is essentially pure (e.g., about 99% by weight or to
homogeneity).

5   Bifunctional molecules which are prepared according
to the above method can be used directly in the
disclosed method or can be screened for activity prior
to use. To screen the bifunctional molecule for
activity, for example, in vitro, the bifunctional
10   molecule (or mixtures of bifunctional molecules) can be
contacted with, for example, the target cell or the
targeted surface protein under conditions suitable for
binding and then assayed for binding. Similarly the
bifunctional molecule can be screened for the ability to
15   bind the viral vector, or the surface protein of the
viral vector, in vitro, by contacting the vector and
bifunctional molecule under conditions suitable for
binding and detecting binding. The bifunctional
molecule can be screened for the ability to induce viral
20   entry by contacting the vector, bifunctional molecule
and target cell under typical cell culture conditions
and detecting infection of the cells (e.g. replication
of viral sequences).

The bifunctional molecule of the invention is
25   particularly useful in the delivery of one or more
polynucleotides (e.g., genes) or products thereof to a
patient. Generally, the polynucleotide is present or
has been incorporated into the genome of the viral
vector. The polynucleotide or the product thereof can
30   be a therapeutic agent. An example of a therapeutic
polynucleotide includes RNA (e.g., ribozymes) and
antisense DNA that prevents or interferes with the

-39-

expression of an undesired protein in the target cell. The polynucleotide can also encode a heterologous therapeutic protein. A heterologous protein or polynucleotide is one which does not exist in the virus

5   as it is found in nature. Examples of therapeutic proteins include antigens or immunogens such as a polyvalent vaccine, cytokines, tumor necrosis factor, interferons, interleukins, adenosine deaminase, insulin, T-cell receptors, soluble CD4, epidermal growth factor,

10   human growth factor, blood factors, such as Factor VIII, Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL receptor, negative selection markers or "suicide proteins", such as thymidine kinase (including the HSV, CMV, VZV TK), anti-angiogenic factors, Fc

15   receptors, plasminogen activators, such as t-PA, u-PA and streptokinase, dopamine, MHC, tumor suppressor genes such as p53 and Rb, monoclonal antibodies or antigen binding fragments thereof, drug resistance genes, ion channels, such as a calcium channel or a potassium

20   channel, and adrenergic receptors, etc.

       The invention is particularly useful for vaccine delivery. In this embodiment, the antigen or immunogen can be expressed heterologously (e.g., by recombinant insertion of a nucleic acid sequence which encodes the

25   antigen or immunogen (including antigenic or immunogenic fragments) in a viral vector). Alternatively, the antigen or immunogen can be expressed in a live attenuated, pseudotyped virus vaccine, for example. Generally, the methods can be used to generate humoral

30   and cellular immune responses, e.g. via expression of heterologous pathogen-derived proteins or fragments thereof in specific target cells.

-40-

Generally, viral vectors which contain therapeutic polynucleotide are known in the art. Examples include the vectors described in Anderson, *et al.* (United States Patent No.5,399,346), Sambrook, *et al.*, *supra*, Ausubel,

5 *et al.*, *supra*, and Weiss, *et al.* (1985) "RNA Tumor Viruses", Cold Spring Harbor, NY, the contents of which are incorporated herein by reference.

The bifunctional molecule of the claimed invention can be employed in a method for delivering a viral

10 vector to a cell comprising contacting the cell with the bifunctional molecule. The cell can be contacted with the viral vector and the bifunctional molecule *in vitro* or *in vivo*. Where the cell is contacted *in vitro*, the target cell containing the viral vector can then be

15 implanted into a patient for delivery of the poly-nucleotide or product thereof. The target cell can be migratory, such as a hematopoietic cell, or non-migratory, such as a solid tumor cell or fibroblast. Frequently, the target cell is targeted from the patient

20 and returned to the patient once contacted with the bifunctional molecule and viral vector. *Ex vivo* gene therapy has been described, for example, in Kasid, *et al. PNAS USA 87*:473 (1990), Rosenberg *et al. N. Engl. J. Med., 323*: 570 (1990), Williams, *et al., Nature 310*:476

25 (1984), Dick, *et al., Cell 42*:71 (1985), Keller, *et al. Nature 318*:149 (1985) and Anderson, *et al.* United States Patent No. 5,399,346.

Alternatively, the bifunctional molecule can be coadministered to the patient with the viral vector.

30 Coadministration is meant to include simultaneous or sequential administration of the two agents, individually or in combination. Where the agents are

-41-

administered individually it is preferred that the mode
of administration is the same and the site of admini-
stration is the same to improve efficiency. It is also
preferred that the administrations are conducted

5   sufficiently close in time so that the active
ingredients are in the presence of the cells
simultaneously. In one embodiment, the bifunctional
molecule is administered prior to the viral vector so
that the molecule can bind to the target cell initially.

10   In another embodiment, the bifunctional molecule is
contacted with the viral vector prior to administration
so that the vector and molecule can bind prior to
contact with the target cell.

The mode of administration is preferably at the

15   location of the target cells. As such, the
administration can be nasally (as in administering a
vector expressing ADA) or by injection (as in
administering a vector expressing a suicide gene to a
tumor). Other modes of administration (parenteral,

20   mucosal, systemic, implant, intraperitoneal, etc.) are
generally known in the art. The agents can, preferably,
be administered in a pharmaceutically acceptable
carrier, such as saline, sterile water, Ringer's
solution, and isotonic sodium chloride solution.

25   The examples presented below are provided as
further guidance and are not to be construed as limiting
the invention in any way.

-42-

Exemplification

EXAMPLE 1

A modified form of a human EGF minigene was
obtained by PCR amplification of AX proviral DNA using
5   the oSS1 and oSS2 primers described below. The modified
form of the human EGF minigene encoded human EGF (Stern,
1987, *Science 235*:322) with a polyproline linker region
(PPPELLGGP) derived from a rabbit Fc chain, added at the
N-terminal end. The PCR product contained an EagI site
10   introduced at the 5' end, and KpnI and EagI sites
following the human EGF stop codon. The PCR product was
digested with the EagI restriction enzyme and introduced
into the EagI site of a synthetic Tva gene (Belanger,
1996, *J. Virol. 69*:1019-1024) contained within the pCI
15   mammalian expression vector (Promega).

oSS1:   gcgcggccgcccacccctgaactcctggggggaccggaggttcag
          aactctgattccgaatgc (SEQ ID NO:1)

oSS2:   gcgcggccgggtaccttatcgcagttccaatttcaggtcgcg
          (SEQ ID NO:2)

20   Human 293 cells grown under standard conditions
(Zingler, *J. Virol. 70*:7510-7516 (1996)) were plated at
20% confluence on 100 mm tissue culture plates. The
cells were transfected with 15µg of the plasmid
expression vector encoding TVA-EGF (described above) by
25   the calcium phosphate transfection procedure (Wigler
Cell 11:223-232 (1977)). Radioactively-labeled TVA-EGF
was prepared by incubating transfected 293 cells with ^{35}S
cysteine: forty eight hours after transfection, the
transfected cells (or for control purposes,

-43-

untransfected cells) were incubated with cysteine/
methionine-free DME (1% dialyzed calf serum) for 1 hour
and then with the same media supplemented with 100μCi/ml
35S-cysteine for 2 hours. The cells were then washed

5   with PBS and incubated with DMEM for 4 hours and the
extracellular supernatants were harvested and stored at
-80°C before analysis. Aliquots of 500μl labeled cell
culture supernatants were incubated with either 12μg of
a subgroup A SU-immunoadhesin (SUA-rIgG)(Zingler, J.

10   Virol. 70:7510-7516 (1996)) or with 26 μg of an antibody
specific for human EGF for 1 hour at 4°C. These samples
were then incubated with protein A-Sepharose, or protein
G-Sepharose, respectively for 1 hour at 4°C. The
precipitated proteins were then collected by

15   centrifugation in a microfuge, the sepharose beads were
washed 4 times each with 1 ml of ice cold PBS and the
samples boiled in a non-reducing protein sample buffer
for 3 minutes. The proteins were then subjected to
electrophoresis on a 10% polyacrylamide gel containing

20   SDS, the gel was then dried and exposed to Kodak XAR-5
film at room temperature. Alternatively, extracellular
supernatants were collected 72 hours after transfection
and 45 μl aliquots subjected to electrophoresis on 10%
polyacrylamide gels containing SDS under non-reducing

25   conditions. Electrophoretically separated proteins were
then transferred to nitrocellulose and the TVA-EGF
detected by immunoblotting with SUA-rIgG followed by
horseradish peroxidase (HRP)-conjugated secondary
antibody specific for rabbit immunoglobulins and

30   enhanced chemiluminescence (Zingler, et al., J. Virol.
70:7510 (1996)). Transiently transfected human 293
cells produced bifunctional molecules containing TVA and

-44-

EGF (TVA-EGF) in the extracellular supernatant whereas
supernatants from untransfected control cells did not
produce TVA-EGF.

EXAMPLE 2

5   Unlabeled TVA-EGF was collected in extracellular
supernatants from 293 cells transfected essentially as
described above except that the supernatants were
harvested 72 hours post-transfection. These
supernatants were filtered through a 0.45 μm filter and
10 stored at -20°C before use. Confluent plates of cells
were washed with Ca^{2+}/Mg^{2+} free PBS and the cells were
removed from the plates with 5 ml of Ca^{2+}/Mg^{2+} free PBS
containing 1 mM EDTA and put on ice. Mouse L cells
lacking EGF receptors (B82), expressing wild type EGF
15 receptors (T23) or expressing EGF receptors lacking in
kinase activity (M5) (Chen, 1987, *Nature 328*:820) (4 x
10^3 cells) were incubated either with unlabeled TVA-EGF
supernatant (0.1, 0.5, 2.5, 12.5, 25, 250 or 500 μl) or
with untransfected 293 cell supernatant for 30 minutes
20 on ice. The cells were spun in a microfuge and washed
with ice cold PBS and incubated with 1 μg of purified
SUA-rIgG or in 1 ml of PBS for 30 minutes on ice. After
washing the cells once with ice cold PBS, the cells were
incubated with 5 μl of FITC-conjugated swine anti-rabbit
25 antibody (DAKO) for 30 minutes on ice. Finally, the
cells were washed once with ice cold PBS and resuspended
in 0.5 ml of PBS containing 1% formaldehyde. The cells
were kept in the dark at 4°C before analysis. Bound
proteins were detected by flow cytometry using a Becton
30 Dickinson FACScan. The TVA-EGF bound subgroups A SU-
immunoadhesin to the M5 and T23 cells that express

-45-

surface EGF receptors (M5 and T23 cells), but not to the
B82 cells which lack the EGF receptor (Figure 2). The
amount of TVA-EGF bound to cells, as measured by mean
fluorescence, increased with increasing amounts of TVA-
5   EGF up to concentrations of 12.5 μg. Amounts of TVA-EGF
higher than 12.5 μg did not result in a significant
increase in the amount of TVA-EGF bound to cells. Thus,
the TVA-EGF bifunctional molecule is capable of dual
binding as indicated by binding to both an EGF receptor
10   on the surface of M5 and T23 target cells and the SUA-
rIgG antibody-like protein.

EXAMPLE 3
         The specificity of TVA-EGF binding was evaluated by
15   competition experiments using recombinant human EGF
(Upstate Biotechnology).
         Bifunctional molecules containing TVA and EGF (TVA-
EGF) produced in the extracellular supernatant from
human 293 cells were collected 72 hours post-
20   transfection as described in Examples 1 and 2. M5 cells
were plated and incubated at 4°C for 1 hour with 200 μl
of extracellular supernatants containing TVA-EGF as
described in Example 2 except that varying amounts of
recombinant human EGF (0-5 μg) were added to the
25   incubation media. The amount of TVA-EGF bound was
determined by flow cytometry detection using SUA-rIgG as
described in Example 2. The amount of TVA-EGF bound to
M5 cells, as measured by mean fluorescence, decreased
with increasing amounts of recombinant EGF (Figure 3).
30   These data show that the binding of TVA-EGF to EGF
receptors was specific for the EGF moiety of the TVA-EGF
bifunctional molecule. As a control, the extracellular

-46-

supernatants of T23TVA cells, which stably express a
transmembrane form of TVA, were also incubated with
increasing amounts of recombinant EGF. T23TVA cells
were generated using previously described procedures
5   (Zingler et al., J. Virol. 69:4261 (1995)). EGF did not
inhibit SUArIgG-TVA binding to T23TVA cells.

    EXAMPLE 4

         As further evidence that the TVA-EGF bifunctional
    molecule binds to the surface of target cells through
10   EGF receptors, the retention of TVA-EGF proteins on the
    surface of M5 and T23 cells was determined.

         M5 and T23 cells were prepared and incubated with
    200 μl of extracellular supernatant containing TVA-EGF
    (4°C, 1 hour) as described in Example 2. Cells were then
15   washed with ice cold PBS and either left on ice (zero
    time point) or incubated with 500 μl of Dulbecco's
    Modified Eagles Medium (DMEM) at 37°C conditions for 30,
    60 or 120 minutes. Following incubation in DMEM, cells
    were placed on ice and the presence of the TVA-EGF
20   bifunctional molecule on the cell surface of M5 and T23
    cells detected by flow cytometry as described in Example
    2. TVA-EGF remained present at the cell surface of M5
    cells, which express EGF receptors lacking kinase
    activity for a longer period of time than to T23 cells,
25   which express wildtype EGF receptors (Figure 4). The
    majority of TVA-EGF present at the cell surface of T23
    cells was cleared from the surface of cells within 30
    minutes whereas a substantial amount of TVA-EGF remained
    on the surface of M5 cells after 120 minutes. These
30   data and the data presented in Figure 3 indicate that
    binding of TVA-EGF to target cells occurs by the EGF

-47-

receptor. Those cells which are deficient in kinase
activity, the M5 cells, retain bound TVA-EGF on their
surface longer than wildtype T23 cells.

EXAMPLE 5

5   M5 cells expressing kinase-deficient EGF receptors
were plated at 10% confluence on 60 mm tissue culture
plates in DME containing 10% dialyzed bovine calf serum
for one day. The plates were then placed on ice, washed
with ice cold PBS, and incubated with 1 ml of either ice
10   cold untransfected 293 cell culture supernatant or with
1 ml ice cold supernatant containing TVA-EGF for 1 hour
at 4°C. The plates were put on ice and washed with ice
cold PBS and then incubated with 1 ml of untransfected
293 cell culture supernatant containing or lacking
15   300 μl of RCASH-A (a subgroup A-specific virus)
containing the hygromycin B-phosphotransferase gene
(Young, 1993, *J. Virol. 67*:1811) (titer 8 x 10^3 IU/ml).
After 1 hour of incubation at 4°C, the plates were
placed in a 37°C incubator and 24 hours later they were
20   selected in medium containing sufficient amounts of
hygromycin B to select against growth of any uninfected
cells (700 μg for M5 cells and 900 μg for B82 cells).
After two weeks of selection, the cells were fixed in
the solution containing 1% (w/v) methylene blue, 20%
25   (v/v) iso-propanol and 5% (v/v) acetic acid and the
colonies were counted. Cells incubated with TVA-EGF
became susceptible to subgroup A viral infection
(Figures 5 and 6). Only M5 cells incubated with
supernatants containing TVA-EGF were infected (Figures 5
30   and 6). TVA-EGF rendered M5 cells highly susceptible
to viral infection. The average number of M5 colonies

-48-

infected with RCASH-A virus was approximately 23% of
that observed after viral infection of T23TVA cells
which express a fully functional epitope-tagged TVA
protein (Figure 6). B82 cells which do not express EGF
5   receptors were not infected following the addition of
TVA-EGF.

EXAMPLE 6
      T23 cells, which express the wildtype EGF; M5
10   cells, which express an EGF receptor lacking kinase
activity; or B82 cells, which lack the EGF receptor,
were plated and incubated with 400 μl of subgroup A ALV
virus containing a neomycin phosphotransferase gene
(RCASA-Neo) at 37°C for 28 hours as described in Example
15   5 except that varying amounts of extracellular
supernatant containing TVA-EGF were added (0, 0.5, 2.5,
12.5 or 200 μl). T23TVA and B82 cells were run in
parallel in infectivity experiments as a positive and
negative controls, respectively. T23TVA cells (positive
20   control) were plated at 4°C for 1 hour, incubated with
1.5 ml of DMEM with or without 50 μl of the RCASA-Neo
virus followed by incubation at 37°C for 28 hours before
selection in medium containing 200 μg/ml Geneticin
(G418). After two weeks of selection, infected cells
25   (G418- resistant colonies) were determined as described
in Example 5. Infectivity increased in M5 and T23 cells
with increasing amounts of TVA-EGF in the incubation
media (Figure 7). The average number of infected cells
observed when M5 cells were incubated with an excess
30   amount of TVA-EGF (200 μl) was approximately 10% of that
obtained with T23TVA cells. The level of infectivity

-49-

obtained with T23 cells was lower than that observed
with M5 cells (Figure 7).

EXAMPLE 7

Infectivity of M5 cells was diminished by the
5   addition of recombinant human EGF (0.5 and 5 μg) (Figure
8). In these experiments M5 cells (1 X 10⁵) were plated
on 60 mm plates and incubated with 1 ml ice cold DMEM
containing 200 μl of TVA-EGF extracellular supernatant
and either 0, 0.5 or 5 μg human recombinant EGF. T23TVA
10   cells were incubated with 1 ml DMEM and either 0 or 5 μg
EGF. After washing in ice cold PBS the cells, 1.5 ml of
DMEM containing 150 μl of RCASA-Neo was added and the
cells were incubated at 37°C for 28 hours. The number of
infected G418 resistant colonies was determined as
15   described in Example 6. Thus, TVA-EGF mediated viral
infection of target cells expressing EGF occurs by
binding of the EGF moiety to EGF cell surface receptors
(Figure 9).

EQUIVALENTS

20   Those skilled in the art will know, or be able to
ascertain, using no more than routine experimentation,
many equivalents to the specific embodiments of the
invention described herein. These and all other
equivalents are intended to be encompassed by the
25   following claims.

-50-

CLAIMS

What is claimed is:

1.   A bifunctional molecule comprising:
      (a)   a first binding moiety which binds to a first
5   cellular surface molecule on a target cell;
            and
      (b)   `a second binding moiety which binds to a viral
            surface molecule on a viral vector and
            activates viral entry, wherein said second
10   binding moiety is an extracellular domain of a
            viral cellular receptor or a derivative
            thereof and said bifunctional molecule is not
            an immunoadhesin.

2.   The bifunctional molecule of Claim 1 wherein the
15   first cell surface molecule is selected from a
      group consisting of carbohydrates, lipids,
      polypeptides, and glycosylated polypeptides.

3.   The bifunctional molecule of Claim 1 wherein the
      first binding moiety is a ligand to a cellular
20   receptor.

4.   The bifunctional molecule of Claim 3 wherein the
      cellular receptor is cell-type specific.

5.   The bifunctional molecule of Claim 4 wherein the
      first binding moiety is a polypeptide.

-51-

6.   The bifunctional molecule of Claim 5 wherein the
      ligand has an amino acid sequence which is the same
      or substantially the same as an amino acid sequence
      of at least the receptor-binding portion of a
5   native ligand for the cellular receptor.

7.   The bifunctional molecule of Claim 5 wherein the
      ligand has an amino acid sequence which is the same
      as an amino acid sequence of at least the receptor-
      binding portion of a native ligand for the cellular
10   receptor.

8.   The bifunctional molecule of Claim 5 wherein the
      target cell is selected from the group consisting
      of epithelial cells, fibroblasts, smooth muscle
      cells, blood cells, tumor cells, cardiac muscle
15   cells, macrophages, dendritic cells, neuronal
      cells, and pathogen-infected cells.

9.   The bifunctional molecule of Claim 5 wherein the
      cellular receptor is selected from the group
      consisting of the subgroup A avian leukosis virus
20   receptor, the subgroup B and D avian leukosis virus
      receptor, the CD4 receptor for human and simian
      immunodeficiency viruses, the amphotropic and
      ecotropic murine leukemia virus receptors, the
      bovine leukemia virus receptor, the gibbon ape
25   leukemia virus receptor, the poliovirus receptor,
      the major subgroup rhinovirus receptor, the
      adenovirus and coxsackie virus receptor, the
      coronavirus receptors, and sialic acid and other
      carbohydrate receptors.

-52-

10. The bifunctional molecule of Claim 5 wherein the
     ligand is selected from the group consisting of
     growth factors, epidermal growth factor,
     interleukins, GM-CSF, G-CSF, M-CSF, EPO, TNF,
5   interferons, and chemokines.

11. The bifunctional molecule of Claim 1 wherein the
     first binding moiety is a single chain antibody
     which binds to a cellular receptor.

12. The bifunctional molecule of Claim 11 wherein the
10   cellular receptor is cell-type specific.

13. The bifunctional molecule of Claim 1 wherein the
     second binding moiety is a polypeptide which has an
     amino acid sequence which is the same or
     substantially the same as an amino acid sequence of
15   at least the viral-binding portion of a native
     extracellular domain of a viral cellular receptor.

14. The bifunctional molecule of Claim 13 wherein the
     second binding moiety has an amino acid sequence
     which is the same as an amino acid sequence of at
20   least the viral-binding portion of a native
     extracellular domain of the viral cellular
     receptor.

15. The bifunctional molecule of Claim 13 wherein the
     native extracellular domain of the viral cellular
25   receptor is on a cell which is subject to infection
     by the viral vector.

-53-

16. The bifunctional molecule of Claim 15 wherein the
    viral vector is selected from the group consisting
    of retroviral vectors, adenoviral vectors,
    parvovirus, coronavirus, orthomyxovirus,
    rhabdovirus, paramyxovirus, picornavirus,
    alphavirus, adenovirus, herpesvirus, poxvirus,
    Norwalk virus, togavirus, flavivirus, reoviruses,
    papovavirus, hepadnavirus, and hepatitis virus.

17. The bifunctional molecule of Claim 15 wherein the
    viral vector is selected from the group consisting
    of a retroviral vector or viral vector which
    comprises a retroviral envelope protein.

18. The bifunctional molecule of Claim 15 wherein the
    viral cellular receptor is epidermal growth factor
    receptor.

19. The bifunctional molecule of Claim 1 further
    comprising a linking moiety.

20. A bifunctional molecule comprising:
    (a) a first binding polypeptide which binds to a
        surface molecule on a target cell, wherein
        said first binding polypeptide is a ligand to
        a cell-type specific cellular receptor;
    (b) a second binding polypeptide which binds to a
        surface molecule on a viral vector, wherein
        the second binding polypeptide is a
        polypeptide which has an amino acid sequence
        which is the same or substantially the same as
        an amino acid sequence of at least the viral-

-54-

binding portion of a native extracellular
domain of a viral cellular receptor.

21.   The bifunctional molecule of Claim 20 wherein the
first cell surface molecule is selected from a
group consisting of carbohydrates, lipids,
polypeptides, and glycosylated polypeptides.

22.   The bifunctional molecule of Claim 20 wherein the
first binding polypeptide and second binding
polypeptide are linked via a peptide bond.

23.   The bifunctional molecule of Claim 22 wherein the
C-terminus of the first binding polypeptide is
linked to the N-terminus of the second binding
polypeptide via a peptide bond.

24.   The bifunctional molecule of Claim 22 wherein the
N-terminus of the first binding polypeptide is
linked to the C-terminus of the second binding
polypeptide via a peptide bond.

25.   The bifunctional molecule of Claim 20 further
comprising a linking moiety.

26.   The bifunctional molecule of Claim 25 wherein the
linking moiety is a polypeptide linker.

27.   The bifunctional molecule of Claim 26 wherein the
C-terminus of the first binding polypeptide is
linked to the N-terminus of the second binding
polypeptide via the polypeptide linker.

-55-

28. The bifunctional molecule of Claim 26 wherein the N-terminus of the first binding polypeptide is linked to the C-terminus of the second binding polypeptide via the polypeptide linker.

5   29. The bifunctional molecule of Claim 26 wherein the polypeptide linker is an immunoglobulin hinge region.

30. The bifunctional molecule of Claim 28 wherein the molecule is expressed from a single nucleic acid
10   molecule.

31. A bifunctional molecule for targeting a retroviral vector to a cell-type comprising:
    (a)   a receptor-binding fragment of a polypeptide ligand which binds a cellular receptor
15   specific to the cell-type;
    (b)   a viral-binding fragment of a cellular receptor for the retroviral vector.

32. The bifunctional molecule of Claim 31 wherein the polypeptide ligand is human epidermal growth
20   factor.

33. The bifunctional molecule of Claim 31 wherein the retroviral vector is avian leukosis virus and the cellular receptor for the retroviral vector is Tva.

34. The bifunctional molecule of Claim 31 further
25   comprising a hinge region linking the receptor-binding fragment to the viral-binding fragment.

-56-

35. A nucleic acid molecule which encodes a
     bifunctional molecule of Claim 1.

36. A nucleic acid molecule which encodes a
     bifunctional molecule of Claim 20.

5 37. The nucleic acid molecule of Claim 36 wherein the
        bifunctional molecule is encoded in a single open
        reading frame.

38. A nucleic acid molecule which encodes a
     bifunctional molecule of Claim 31.

10 39. The nucleic acid molecule of Claim 38 wherein the
        bifunctional molecule is encoded in a single open
        reading frame.

40. A method for preparing a bifunctional molecule
     comprising:

15   (a) a first binding moiety which binds to a
             surface
             molecule on a target cell; and
        (b) a second binding moiety which binds to a
             surface molecule on a viral vector, wherein
20   said second binding moiety is an extracellular
             domain of a viral cellular receptor or a
             derivative thereof and said bifunctional
             molecule is not an immunoadhesin;
     wherein the method comprises maintaining a host
25   cell which expresses the bifunctional molecule
     under conditions suitable for expression and
     recovering the bifunctional molecule.

-57-

41.   The method of Claim 40 wherein the first cell
      surface molecule is selected from a group
      consisting of carbohydrates, lipids, polypeptides,
      and glycosylated polypeptides.

5   42.   A method for preparing a bifunctional molecule
      comprising conjugating a first binding moiety which
      binds to a surface protein on a target cell and a
      second binding moiety which binds to a surface
      protein on a viral vector, wherein said second
10   binding moiety is an extracellular domain of a
      viral cellular receptor or a derivative thereof.

43.   A method for delivering a viral vector to a cell
      comprising contacting the cell with the viral
      vector and a bifunctional molecule comprising:
15   (a)   a first binding moiety which binds to a
            surface molecule of the target cell; and
      (b)   a second binding moiety which binds to a
            surface molecule on a viral vector, wherein
            said second binding moiety is an extracellular
20   domain of a viral cellular receptor or a
            derivative thereof.

44.   The method of Claim 43 wherein the first cell
      surface molecule is selected from a group
      consisting of carbohydrates, lipids, polypeptides,
25   and glycosylated polypeptides.

45.   The method according to Claim 43 wherein the cell
      is contacted with the viral vector in vitro.

46. The method according to Claim 43 wherein the cell is contacted with the viral vector *in vivo*.

47. The method according to Claim 43 wherein the viral vector encodes a heterologous protein.

5   48. The method according to Claim 47 wherein the heterologous protein is selected from the group consisting of immunogens, cytokines, tumor necrosis factor, interferons, interleukins, adenosine deaminase, insulin, T-cell receptors, soluble CD4,

10   epidermal growth factor, human growth factor, blood factors, such as Factor VIII, Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL receptor, negative selection markers, anti-angiogenic factors, Fc receptors, plasminogen

15   activators, dopamine, MHC, tumor suppressor genes, monoclonal antibodies or antigen binding fragments thereof, drug resistance genes, ion channels and adrenergic receptors.

49. A method for delivering a viral vector to a cell

20   comprising contacting the cell with the viral vector and a bifunctional molecule comprising:

(a)   a first binding polypeptide which binds to a surface molecule on a target cell, wherein said first binding polypeptide is a ligand to

25   a cell-type specific cellular receptor;

(b)   a second binding polypeptide which binds to a surface molecule on a viral vector, wherein the second binding polypeptide is a polypeptide which has an amino acid sequence

which is the same or substantially the same as an amino acid sequence of at least the viral-binding portion of a native extracellular domain of a viral cellular receptor.

5 50. The method of Claim 49 wherein the first cell surface molecule is selected from a group consisting of carbohydrates, lipids, polypeptides, and glycosylated polypeptides.

51. A method for delivering a retroviral vector to a

10 cell comprising contacting the cell with the retroviral vector and a bifunctional molecule comprising:

    (a) a receptor-binding fragment of a polypeptide ligand which binds a cellular receptor

15   specific to the cell-type;

    (b) a viral-binding fragment of a cellular receptor for the retroviral vector.

52. The method according to Claim 51 wherein the cell is contacted with the retroviral vector *in vitro*.

20 53. The method according to Claim 51 wherein the cell is contacted with the retroviral vector *in vivo*.

54. The method according to Claim 51 wherein the retroviral vector encodes a heterologous protein.

55. The method according to Claim 54 wherein the

25 heterologous protein is selected from the group consisting of immunogens, cytokines, tumor necrosis

factor, interferons, interleukins, adenosine
deaminase, insulin, T-cell receptors, soluble CD4,
epidermal growth factor, human growth factor, blood
factors, such as Factor VIII, Factor IX, cytochrome
b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL
receptor, negative selection markers, anti-
angiogenic factors, Fc receptors, plasminogen
activators, dopamine, MHC, tumor suppressor genes,
monoclonal antibodies or antigen binding fragments
thereof, drug resistance genes, ion channels and
adrenergic receptors.

56. The method according to Claim 54 wherein the
polypeptide ligand is human epidermal growth
factor.

57. The method according to Claim 56 wherein the
retroviral vector is avian leukosis virus and the
cellular receptor for the retroviral vector is Tva.

58. The method according to Claim 57 wherein the
bifunctional molecule further comprising a hinge
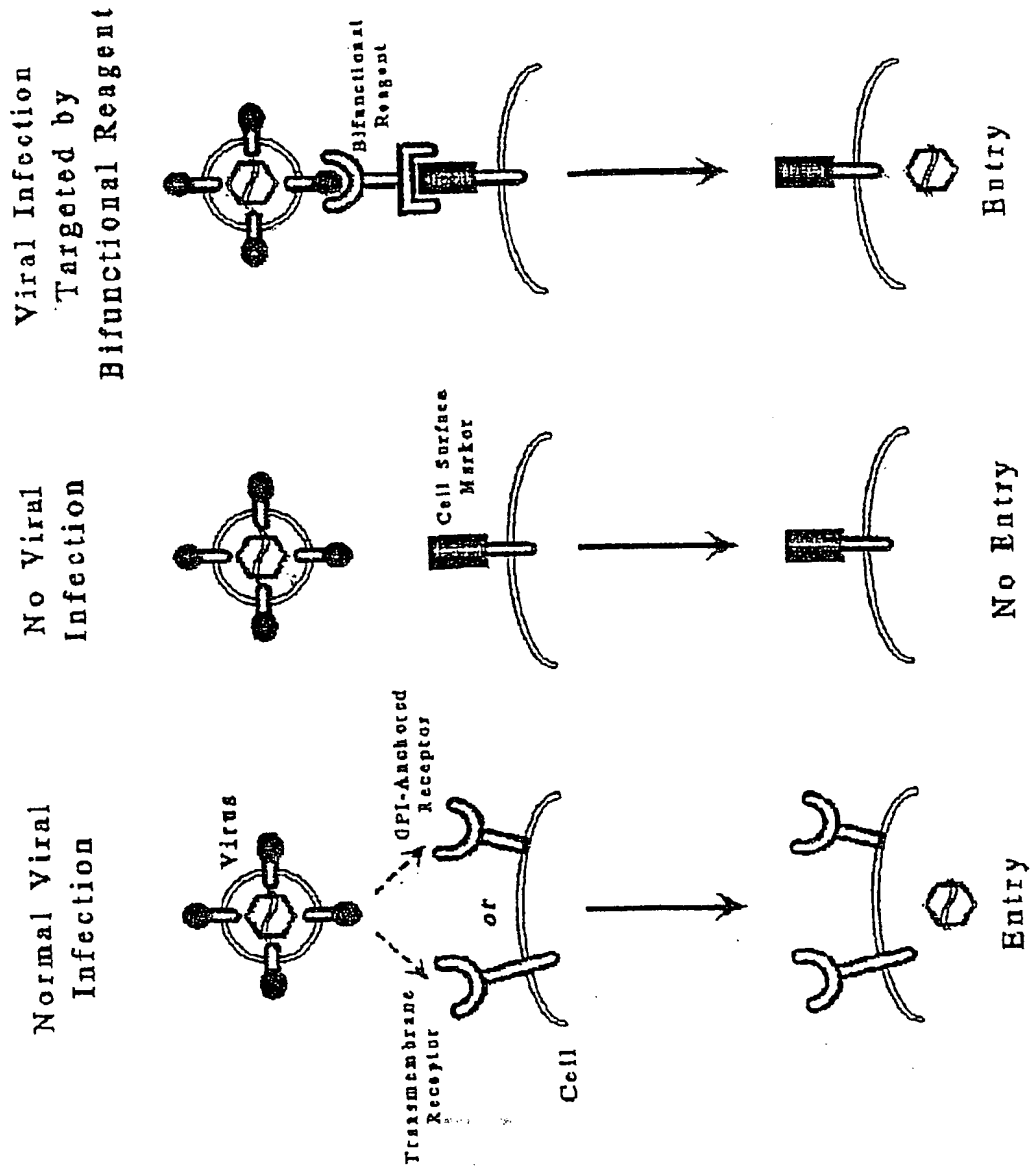region linking the receptor-binding fragment to the
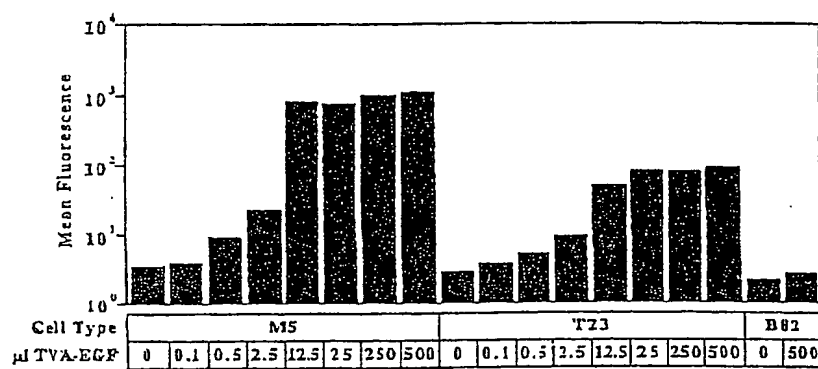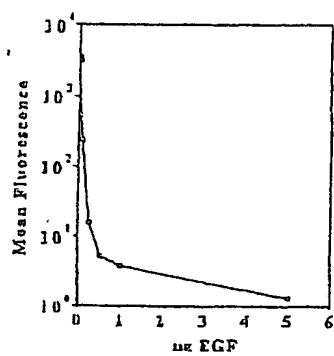viral-binding fragment.

FIGURE 1

Cell Type	M5								T23								B82	
μl TVA-EGF	0	0.1	0.5	2.5	12.5	25	250	500	0	0.1	0.5	2.5	12.5	25	250	500	0	500

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

Cell Type	T23-TVA	MS					T23					B82
µl TVA-EGF	0	200	12.5	2.5	0.5	0	200	12.5	2.5	0.5	0	200
µl RCASA-Neo	50	400										

FIGURE 7

FIGURE 8

ALV-A
Env

TVA

EGF

EGFR

FIGURE 9

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C07K14/00   C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 27643 A (TARGETED GENETICS CORP ;PAUL RALPH W (US); OVERELL ROBERT (US)) 8 December 1994 see page 7, line 22 - page 9, line 23 see page 13, line 31 - page 16, line 28 see page 38, line 1 - page 40, line 5 ---	1-58
X	FR 2 649 119 A (CENTRE NAT RECH SCIENT) 4 January 1991 see page 1, line 31 - page 2, line 25 see claims 1-15 ---	1-58
X	WO 93 25682 A (UNIV NEW YORK ;MERUELO DANIEL (US); YOSHIMOTO TAKAYUKI (JP)) 23 December 1993 see page 51, line 27 - page 52, line 15 ---	1-58

-/--

| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 September 1998	06/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Seegert, K

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication.where appropriate. of the relevant passages	Relevant to claim No.
X	WO 93 09221 A (THERA GENE HB) 13 May 1993 see page 1, line 30 - page 2, line 13 ---	1-58
X	ETIENNE-JULAN M ET AL: "THE EFFICIENCY OF CELL TARGETING BY RECOMBINANT RETROVIRUSES DEPENDS ON THE NATURE OF THE RECEPTOR AND THE COMPOSITION OF THE ARTIFICIAL CELL-VIRUS LINKER" JOURNAL OF GENERAL VIROLOGY, vol. 73, December 1992, pages 3251-3255, XP000612121 see abstract -----	1-58

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9427643	A	08-12-1994	AU	7097494 A	20-12-1994
			US	5736387 A	07-04-1998
FR 2649119	A	04-01-1991	NONE		
WO 9325682	A	23-12-1993	AU	4532293 A	04-01-1994
			CA	2137547 A	23-12-1993
			EP	0644936 A	29-03-1995
			JP	7509599 T	26-10-1995
WO 9309221	A	13-05-1993	SE	503225 C	22-04-1996
			CA	2122613 A	13-05-1993
			EP	0614486 A	14-09-1994
			JP	7502026 T	02-03-1995
			SE	9103183 A	01-05-1993
			US	5695991 A	09-12-1997

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : **C07K 14/00, C12N 15/62**	**A1**	(11) International Publication Number: **WO 98/47916** (43) International Publication Date: 29 October 1998 (29.10.98)

(21) International Application Number: PCT/US98/07720

(22) International Filing Date: 16 April 1998 (16.04.98)

(30) Priority Data:
08/844,359   18 April 1997 (18.04.97)   US

(63) Related by Continuation (CON) or Continuation-in-Part
(CIP) to Earlier Application
US   08/844,359 (CIP)
Filed on   18 April 1997 (18.04.97)

(71) Applicant *(for all designated States except US)*: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 1350 Massachusetts Avenue, Cambridge, MA 02138 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: YOUNG, John [GB/US]; 388 Lexington Street, Newton, MA 02166 (US). SNITKOVSKY, Sophie [—/US]; 382 Ocean Avenue #401, Revere, MA 02151 (US).

(74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).

(81) Designated States: AU, CA, JP, NZ, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BIFUNCTIONAL POLYPEPTIDES FOR CELL–SPECIFIC VIRAL TARGETING

(57) Abstract

The invention relates to a novel bifunctional molecule comprising a first binding moiety which binds to a surface molecule on a target cell; and a second binding moiety which binds to a surface molecule on a viral vector. The bifunctional molecule targets the viral vector to the target cell with improved infectivity and selectivity. The molecule can be used, for example, in *in vitro* and *in vivo* gene delivery methods.

BIFUNCTIONAL POLYPEPTIDES
FOR CELL-SPECIFIC VIRAL TARGETING

RELATED APPLICATION

This application is a continuation-in-part of U.S.
5 Serial No. 08/844,359, filed April 18, 1997, the
teachings of which are incorporated herein by reference,
in their entirety.

GOVERNMENT FUNDING

The invention described herein was supported in
10 whole or in part by Grant No. R29CA6200 from the
National Institutes of Health. The government has
certain rights in the invention.

BACKGROUND OF THE INVENTION

Many clinical trials for gene delivery have been
15 and are presently employing viral vectors which include
in their genome a nucleic acid sequence encoding a
desired protein. Viral vectors which have been developed
for gene delivery include retroviruses, adenoviruses and
adeno-associated viruses. In recent years, significant
20 advances have occurred in the development of viral
vectors for gene delivery to increase infectivity and
decrease their ability to replicate *in vivo*, for
example.

It has been recognized that it is desirable in some
25 instances to target the viral vector to a specific cell
type. Cell-specific targeting has been suggested for
retroviral vectors. These viruses contain two envelope
glycoprotein subunits designated surface (SU) and

transmembrane (TM) which form an oligomeric complex on
the viral surface and mediate viral entry. The SU
protein contains the viral receptor binding determinants
whereas the TM protein contains a hydrophobic
5   transmembrane region and a separate hydrophobic segment
that mediates virus-cell membrane fusion (Weiss, R.A.,
1993, "Cellular receptors and viral glycoproteins
involved in retrovirus entry," p. 1-107, in J.A. Levy
(ed.), *The Retroviridae*, Vol. 2. Plenum press, New
10   York).

To date, there have been several reports of cell
type-specific retrovirus targeting. In one case,
retroviral particles were chemically modified with
lactose, a procedure which resulted in specific
15   infection of human cells that expressed the asialogly-
coprotein receptor (Neda, H., 1991, *J. Biol. Chem.*
266:14143-14149). In another case, a low level of cell
type-specific viral infection was achieved by forming an
antibody bridge between a retroviral SU protein and
20   specific host cell surface proteins (Roux, P., 1989,
Proc. Natl. Acad. Sci. USA 86:9079). An alternative
strategy that has been employed reengineered retroviral
SU proteins to contain cell type-specific ligands or
single chain antibodies that bind to specific cell
25   surface proteins (Valsesia-Wittmann, S., 1994, *J. Virol.*
68:4609-4619; Kasahara, Y., 1994, *Science 266*:1373-1376;
Han, X., 1995, *Proc. Natl. Acad. Sci. USA 92*:9747-9751;
Matano, T., 1995, *J. Gen. Virol.* 76:3165-3169; Valsesia-
Wittmann, 1996, *J. Virol.* 70:2059-2064; Cosset, F-L.,
30   1995, *J. Virol.* 69:6314-6322; Nilson, B.H.K., 1996, *Gene*
Therapy 3:280-286; Etienne-Julan, M., 1992, *J. Virol.*
69:2659-2663; Somia, N., 1995, *Proc. Natl. Acad. Sci USA*

-3-

92:7570-7574; Russell, S.J., 1993, *Nucleic Acids Res.*
21:1081-1085; Marin, M., 1996, *J. Virol.* 70:2957-2962).
This latter approach requires a) the synthesis of
recombinant envelope-ligand proteins in virus producing
5   cells, b) the incorporation of the recombinant ligand-
envelope fusion proteins (and in some cases also wild-
type envelope proteins) onto viral surfaces, c) binding
of the recombinant ligand-envelope fusion to a specific
cell surface receptor, and d) fusion of the viral
10   membrane with the host cell membrane to allow infection.

    The current strategies for retroviral vector
targeting suffer from a number of limitations which
severely restrict the utility of these vectors for a
number of gene therapy approaches. These problems are
15   discussed in Cosset, F-L and Russell, S.J., 1996, *Gene
Therapy* 3:946-956. These problems include the fact that
it is difficult to manipulate these proteins "without
impairing some critical envelope functions;" "Only a few
variable peptides or loops in the SU glycoprotein have
20   been found to tolerate substitution by foreign
peptides;" in some cases the need for co-expressed wild-
type envelope proteins "has not been adequately
explained and greatly complicates the process whereby
targeted vector particles are generated;" in general
25   these strategies give rise to viruses that infect cells
with a low efficiency (Cosset, 1996, *Gene Therapy* 3:946-
956).

    Thus, there is a continued need to develop new and
improved methods for targeting viral vectors to specific
30   cell types.

-4-

SUMMARY OF THE INVENTION

This invention is based upon the discovery that
bifunctional molecules, e.g., polypeptides, comprising a
specific binding and entry moiety, or partner, to a
5   viral vector and a specific binding moiety, or partner,
to a target cell results in efficient binding and
infection of the cell and, thereby, gene delivery. Thus
the invention relates to a bifunctional molecule
comprising a first binding moiety which binds to a
10   surface protein on a target cell, a second binding
moiety which binds to a surface protein on a viral
vector and activates viral entry and, optionally, a
linking moiety. In a preferred embodiment, the first
binding polypeptide is a ligand to a cell-type specific
15   cellular receptor and/or the second binding polypeptide
is a polypeptide which has an amino acid sequence which
is the same or substantially the same as an amino acid
sequence of at least the viral-binding portion or
fragment of a native extracellular domain of a viral
20   cellular receptor.

In another embodiment the first binding moiety
binds to a cell surface molecule on a target cell and
the second binding moiety to a viral surface molecule.
The target cell surface molecule can be a peptide,
25   sugar, lipid, anion or cation, or any combination
thereof which are required for specific, high affinity
binding and activate viral entry into the target cell.

The invention also includes nucleic acid molecules
which encode, independently or together, polypeptide
30   binding moieties of the above bifunctional molecule.
Also envisioned are host cells which comprise the
nucleic acid molecules of the invention and express the

polypeptide binding moieties separately or together as a
bifunctional molecule.

In yet another embodiment, the invention includes
methods for preparing the bifunctional molecules of the
5   invention which includes maintaining one or more
recombinant host cells which express the bifunctional
molecule (e.g., which contain one or more nucleic acid
molecules encoding the bifunctional molecule) under
conditions suitable for expression. Alternatively, the
10 bifunctional molecules can be prepared via chemical
syntheses, such as conjugating the two binding moieties.

The invention also includes methods for delivering
a viral vector, such as a retroviral vector, to a cell,
in vitro or in vivo, comprising contacting the cell with
15 a bifunctional molecule, as described herein. The
retroviral vector typically encodes a heterologous
protein.

In yet another embodiment, the invention relates to
methods of screening ligand and ligand-cell specific
20 receptor binding pairs using bifunctional molecules.

The inventions which are described herein provide
alternative and improved mechanisms for the successful
gene delivery to specific cell types via viral vectors.
In contrast to the prior art, the present invention does
25 not require the careful incorporation of a cellular
binding partner into the viral particle without
disturbing the conformation of a viral protein (e.g. the
env protein) required for proper binding and cell entry.
Neither does it require the specific cloning and
30 elucidation of the viral protein required for binding
and the mechanisms for infection. The invention thereby

-6-

offers greater opportunities in cell-type specific
targeting than previously offered in the prior art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically illustrates the use of the
5   bifunctional molecule in binding and inducing infection
of a cell.

Figure 2 is a bar graph which illustrates TVA-EGF
binding to mouse L cells expressing EGF receptors
lacking kinase activity (M5) and wild type EGF receptors
10   (T23) but not to mouse L cells lacking EGF receptors
(B82).

Figure 3 is a line graph which illustrates the EGF
receptor specificity of TVA-EGF cell surface binding to
M5 cells.

15   Figure 4 is a three dimensional scatter diagram
which illustrates differences in the time required for a
bifunctional molecule containing TVA and EGF to be
cleared from the cell surface of M5 and T23 cells.

Figure 5 is a bar graph which illustrates the avian
20   leukosis viral (ALV) infection of M5 cells which express
the epidermal growth factor EGF receptor with a
bifunctional molecule containing TVA and EGF (TVA-EGF).

Figure 6 is a bar graph which illustrates TVA-EGF
dependent ALV-A infection of M5 cells, which express the
25   EGF receptor, but not B82 cells, which do not express
the EGF receptor.

Figure 7 is a bar graph which illustrates TVA-EGF
dose dependent effects on ALV-A infectivity of T23 and
M5 cells but not in B82 cells.

-7-

Figure 8 is a bar graph which illustrates the EGF receptor specificity of TVA-EGF mediated ALV-A infection in M5 cells.

Figure 9 schematically illustrates binding of the
5   TVA-EGF bifunctional molecule to the EGF receptor on a target cell (EGF binding moiety) and the Env protein of a viral vector (TVA binding moiety).

DETAILED DESCRIPTION OF THE INVENTION

As set forth above, the present invention relates
10 to the discovery that viral vectors can be targeted to specific cell-types by contacting the viral vectors and cells with bifunctional molecules that possess moieties which bind to surface proteins of both the vector and cell. The bifunctional molecule thus comprises a first
15 binding moiety which binds to a surface protein on a target cell and a second binding moiety which binds to a surface protein on a viral vector and induces viral entry.

Viral vectors include retrovirus, adenovirus,
20 parvovirus (e.g., adeno-associated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e.g., influenza virus), rhabdovirus (e.g., rabies and vesicular stomatitis virus), paramyxovirus (e.g. measles and Sendai), positive strand
25 RNA viruses such as picornavirus and alphavirus, and double stranded DNA viruses including adenovirus, herpesvirus (e.g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e.g., vaccinia, fowlpox and canarypox). Other viruses
30 include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis

-8-

virus, for example. Examples of retroviruses include:
avian leukosis-sarcoma, mammalian C-type, B-type
viruses, D-type viruses, HTLV-BLV group, lentivirus,
spumavirus (Coffin, J.M., 1996, Retroviridae: The
5   viruses and their replication. in Fundamental Virology,
Third Edition, edited by B.N. Fields, D.M. Knipe, P.M.
Howley, et al. Lippincott-RavenPublishers,
Philadelphia). Other examples include murine
leukemia viruses, murine sarcoma viruses, mouse mammary
10 tumor virus, bovine leukemia virus, feline leukemia
virus, feline sarcoma virus, avian leukemia virus, human
T-cell leukemia virus, baboon endogenous virus, Gibbon
ape leukemia virus, Mason Pfizer monkey virus, simian
immunodeficiency virus, simian sarcoma virus, Rous
15 sarcoma virus and lentiviruses.

Retroviruses are a family of RNA viruses which
infect cells in a two step mechanism. The first step of
infection is the binding of the viral particle via the
surface protein of the retrovirus envelope (env) protein
20 and viral and cellular membrane fusion for viral uptake
via the transmembrane protein of the env protein. This
is discussed in more detail above. The env protein is
largely responsible for the specificity (between cell-
types and between species) of the infectivity of
25 retroviral vectors.

Adenoviruses have a linear double-stranded DNA
genome. Adenoviruses infect cells by a two step
mechanism. First a viral surface fiber protein binds
specifically to a cell surface receptor. In the case of
30 human HeLa cells, the receptor for adenoviruses 2 and 5
is designated CAR, a member of the immunoglobulin
protein superfamily, which also serves as a cellular

-9-

receptor for coxsackie B viruses (Bergelson, 1996, *Science*, *275*:1320-1323). However, other viral receptors have been described. Following receptor binding, adenoviruses are taken up into the cell by receptor-

5 mediated endocytosis and interaction between the viral penton base protein and cellular integrins is necessary for viral entry (Wickham, 1993, *Cell 73*:309; Bai, 1994, *J. Virol 68*:5925; Goldman, 1995 *J. Virol. 69*:5951; Huang, 1996, *J. Virol. 70*:4502). The viral DNA is

10 replicated in the cell extrachromosomally (Horwitz, M.S., 1996 "Adenoviruses," in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

Recombinant adenoviral vectors are generated by a

15 variety of techniques that include introducing the desired gene of interest into a bacterial plasmid at a site flanked by adenovirus sequences. These sequences provide control elements for gene expression and serve as sites for recombination with a compatible adenoviral

20 genome when cotransfected together into an appropriate mammalian cell line (Horwitz, M.S., 1996 "Adenoviruses," in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

25 Adeno-associated viruses (AAV) have a linear single-stranded DNA genome and their receptor has not yet been described. These viruses only undergo productive infection if the infected cells are coinfected with a helper virus (e.g., adeno- or

30 herpesvirus) otherwise the genome becomes integrated in a latent state at a specific site on a human chromosome (Linden, 1996, *Proc. Natl. Acad. Sci. USA 93*:11288-

11294; Berns, K.J., "Parvoviridae: The viruses and their replication" in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA). Adeno-associated

5   viral vectors are typically made by replacing viral genes with desired genes of interest or instead by simply adding the terminal AAV DNA sequences (ITRs) to these genes.

The negative strand RNA viruses infect cells by a

10 variety of different mechanisms. For example, Influenza A viruses which have a segmented RNA genome, contain a surface hemagglutinin protein which binds to cell surface sialic acid receptors and mediates viral entry in a low pH endosome following receptor-mediated

15 endocytosis (Lamb, R.A. and Krug, R.M., 1996, "Orthomyxoviridae: The viruses and their replication" *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

20   Paramyxoviruses which have a non-segmented RNA genome have two surface viral proteins, the hemagglutinin (HN) and fusion protein (F), required for viral entry which occurs at neutral pH. These viruses can utilize sialic acid receptors, or protein receptors

25 (e.g., CD46 used by measles virus), for viral entry (Lamb, R.A. and Kolakofsky, D., 1996, "Paramyxoviridae: The viruses and their replication" *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers,

30 Philadelphia, PA).

Rhabdoviruses (e.g., VSV) which have a non-segmented RNA genome, contain a surface protein (G)

-11-

which binds to specific cell surface receptors and
mediates viral entry in a low pH endosome. A specific
phospholipid appears to be one of the receptors for VSV
(Wagner, R.R. and Rose, J.K., 1996, in *Fields Virology*,
5 Third Edition edited by B.N. Fields, D.M. Knipe, P.M.
Howley *et al.*, Lippincott-Raven Publishers,
Philadelphia, PA).

A number of strategies are currently available for
engineering negative strand viral RNA genomes to express
10 heterologous genes (reviewed by Palese, P., 1996, *Proc.
Natl. Acad. Sci. USA 93*:11354-11358; Schnell, M.J.,
1996, *Proc. Natl. Acad. Sci. USA 93*:11359-11365).

The positive strand RNA viruses also infect cells
by a variety of different mechanisms. For example,
15 among the picornaviruses, different members of the
immunoglobulin protein superfamily are used as cellular
receptors by poliovirus, by the major subgroups of
rhinoviruses, and by coxsackie B viruses, whereas an
integrin protein is used by some types of ecoviruses and
20 a low density lipoprotein receptor is used by minor
subgroups of rhinoviruses (Bergelson, 1996, *Science
275*:1320-1323; Rueckert, R.R., 1996, "Picornaviridae:
The viruses and their replication" *Fields Virology*,
Third Edition edited by B.N. Fields, D.M. Knipe, P.M.
25 Howley *et al.*, Lippincott-Raven Publishers,
Philadelphia, PA). Following receptor-binding, it is
not yet known precisely what role receptor-mediated
endocytosis plays for picornaviral entry, if indeed it
is required.

30   Because the picornaviruses lack a surface lipid
bilayer, their entry pathway does not involve fusion of
a viral membrane with a host cell membrane. In

-12,-

contrast, the alphaviruses (e.g., Sindbis virus and
Semliki virus) do contain a surface lipid bilayer.
These viruses contain two (E1 and E2) surface proteins,
and in some cases a third (E3) surface protein important
5   for viral entry. These viruses use various cell surface
receptors. For example, Sindbis virus can use a laminin
receptor or other receptors and generally enter cells by
a pH-dependent mechanism, following receptor-mediated
endocytosis (Frolov, 1996, *Proc. Natl. Acad. Sci. USA*
10 *93*:11371-11377; Schlesinger, S. and Schlesinger, M.J.,
1996, "Togaviridae: The viruses and their replication,"
in *Fields Virology*, Third Edition edited by B.N. Fields,
D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven
Publishers, Philadelphia, PA).

15   The herpesviruses which have large double-stranded
DNA genomes, contain a number of surface glycoproteins
involved in viral entry and utilize various cell surface
receptors. For example, herpes simplex virus and
cytomegalovirus entry involves binding to a heparin
20 sulfate cell surface receptor and herpes simplex viruses
use other proteins (e.g., HVEM) for viral entry
(Montgomery, R., 1996, *Cell 87*:427-436). In contrast,
Epstein-Barr virus entry is initiated by binding to a
completely distinct cell surface receptor, CR2 (Wolf,
25 1993, *Intervirology 35*:26-39). Strategies have been
described that allow one to engineer herpes simplex
viruses, cytomegaloviruses and Epstein-Barr viruses as
vectors for heterologous gene expression (Roizman, 1996,
Proc. Natl. Acad. Sci. USA 93:11307-11312; Andreansky,
30 1996, *Proc. Natl. Acad. Sci. USA 93*:11313-11318;
Marconi, 1996, *Proc. Natl. Acad. Sci. USA 93*:11319-
11320; Mocarski, 1996, *Proc. Natl. Acad. Sci. USA*

-13-

93:11321-11326; Robertson, 1996, *Proc. Natl. Acad. Sci.
USA 93*:11334-11340; Duboise, 1996, *Proc. Natl. Acad.
Sci. USA 93*:11389-11394).

Poxviruses have large double stranded DNA genomes
5 and enter cells by a pH-independent mechanism via
receptors that remain to be defined (Moss, B., 1996:
"Poxviridae: The viruses and their replication," in
Fields Virology, Third Edition edited by B.N. Fields,
D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven
10 Publishers, Philadelphia, PA). Poxvirus vectors have
been used extensively for the expression of heterologous
recombinant genes and as vaccines (Moss, B., 1996, *Proc.
Natl. Acad. Sci. USA 93*:11341-11348; Paoletti, 1996,
Proc. Natl. Acad. Sci. USA 93:11349-11353).

15 Viral vectors which have been "pseudotyped"
incorporate the env protein from a retrovirus of a
desired specificity into the vector. Thus, a
pseudotyped virus has the env protein from a first
retrovirus and core or structural proteins from a second
20 virus (e.g. a second retrovirus, an orthomyxovirus or a
rhabdovirus). Viral pseudotypes have been described,
for example, in Le Guen, 1992, *Proc. Natl. Acad. Sci.
USA 89*:363-367; Rizvi, 1992, *Journal of Medical
Primatology 21*:69-73; Takeuchi, 1992, *Virology 186*:792-
25 294; Vile *et al.*, 1991, *Virology 180*:420; Miller *et al.*,
1991, *J. Virol. 65*:2220; Landau, *et al.*,, 1991, *J.
Virol. 65*:162; Emi, *et al.*, 1991, *J. Virol. 65*:1207; and
Dong, *et al.*, 1992, *J. Virol 66*:7374. The present
invention can thus target a pseudotyped viral vector
30 with a bifunctional molecule which binds to the target
cell surface protein and binds to the heterologous env
protein, inducing viral entry.

Typically, retroviral vectors are manufactured by "packaged cell lines" which provide the retroviral proteins necessary for infection (e.g., env, gag and pol), but are incapable of replication upon infection.

5   See, for example, Miller, AD, 1992, Current Topics in Microbiology and Immunology, Vol. 158, pages 1-24.

The viral vectors employed in the present invention can be used for polynucleotide or gene delivery to a cell or animal. The polynucleotide to be delivered to

10   the cell or animal can include a polynucleotide native to the viral vector or heterologous to the vector. In a preferred embodiment, the viral vector has been engineered to contain a polynucleotide which is itself therapeutic or encodes a therapeutic protein, which will

15   be discussed in more detail below.

The invention, as provided for herein, is a mechanism by which the viral vector can more efficiently deliver a desired gene to a cell or animal by indirectly binding the vector to the target cell via a bifunctional

20   molecule comprising a first and second binding moiety.

A "binding moiety," as that term is defined herein, includes a chemical entity which binds to the specified target molecule. The binding can be via a covalent bond, ionic bonding, hydrogen bonding or other

25   mechanism. The binding moiety can be a peptide (including post-translationally modified proteins, such as amidated, demethylated, glycosylated or phosphor-ylated proteins), sugar, lipid, steroid, nucleic acid, small molecule, anion or cation, or combination thereof

30   which binds the specified target molecule. Preferably, the binding will possess a high affinity. Examples of

-15-

high affinity, as that term is used herein, have an
equilibrium constant of 10^{-6} (preferably 10^{-8}) or lower.

As set forth above, the first binding moiety binds
a surface protein, or target molecule, on the target

5   cell. The "target cell" is defined as the cell which is
intended to be infected by the viral vector. Typically,
the target cell is of animal origin and can be a stem
cell or somatic cells. Suitable animal cells for use on
the claimed invention can be of, for example, mammalian

10 and avian origin. Examples of mammalian cells include
human, bovine, ovine, porcine, murine, rabbit cells.
The cell may be an embryonic cell, bone marrow stem cell
or other progenitor cell. Where the cell is a somatic
cell, the cell can be, for example, an epithelial cell,

15 fibroblast, smooth muscle cell, blood cell (including a
hematopoietic cell, red blood cell, T-cell, B-cell,
etc.), tumor cell, cardiac muscle cell, macrophage,
dendritic cell, neuronal cell (e.g., a glial cell or
astrocyte), or pathogen-infected cell (e.g., those

20 infected by bacteria, viruses, virusoids, parasites, or
prions).

Typically, cells isolated from a specific tissue
(such as epithelium, fibroblast or hematopoietic cells)
are categorized as a "cell-type." The cells can be

25 obtained commercially or from a depository or obtained
directly from an animal, such as by biopsy.
Alternatively, the cell need not be isolated at all from
the animal where, for example, it is desirable to
deliver the vector to the animal in gene therapy.

30 Cells are typically characterized by markers
expressed at their surface that are termed "surface
markers". These surface markers include surface

proteins or target molecules, such as cellular
receptors, adhesion molecules, transporter proteins,
components of the extracellular matrix and the like.
These markers, proteins and molecules also include

5   specific carbohydrates and/or lipid moieties, for
example, conjugated to proteins. The first binding
moiety binds to one or more surface proteins on the
target cell. Surface proteins can be tissue- or cell-
type specific (e.g. as in surface markers) or can be

10   found on the surface of many cells. Typically, the
surface marker, protein or molecule is a transmembrane
protein with one or more domains which extend to the
exterior of the cell (e.g. the extracellular domain).
Where cell-type specific delivery is desired (as in *in*

15   *vivo* delivery of a viral vector), the surface protein
selected for the invention is preferably specific to the
tissue. By "specific" to the tissue, it is meant that
the protein be present on the targeted cell-type but not
present (or present at a significantly lower concentra-

20   tion) on a substantial number of other cell-types.
While it can be desirable, and even preferred, to select
a surface protein which is unique to the target cell, it
is not required for the claimed invention. It is to be
appreciated, however, that specific delivery may not be

25   required where the cell or cells are contacted with the
viral vector in pure or substantially pure form, such as
can be the case in an *in vitro* gene transfer. As such,
the surface protein or targeted protein for the first
binding moiety may be present on many different cell-

30   types, specific or even unique to the targeted cell-
type.

-17-

As set forth above, the surface protein can be a cellular receptor or other protein, preferably a cellular receptor. Examples of cellular receptors include receptors for cytokines, growth factors, and

5   include, in particular epidermal growth factor receptors, platelet derived growth factor receptors, interferon receptors, insulin receptors, proteins with seven transmembrane domains including chemokine receptors and frizzled related proteins (Wnt receptors),

10 immunoglobulin-related proteins including MHC proteins, CD4, CD8, ICAM-1, etc., tumor necrosis factor-related proteins including the type I and type II TNF receptors, Fas, DR3, DR4, CAR1, etc., low density lipoprotein receptor, integrins, and, in some instances, the Fc

15 receptor.

Other examples of surface proteins which can be used in the present invention include cell-bound tumor antigens. Many of these surface proteins are commercially available and/or have been characterized in

20 the art, including the amino acid and nucleic acid sequences, which can be obtained from, for example, GENBANK, as well as the specific binding characteristics and domains. Cytokine and chemokine receptors are reviewed for example, in Miyama, *et al. Ann. Rev.*

25 *Immunol., 10*:295-331 (1992), Murphy, *Ann. Rev. Immunol. 12*:593-633 (1994) and Miller *et al. Critical Reviews in Immunol. 12*:17-46 (1992).

Typically, the first binding moiety is selected or derived from native ligands or binding partners to the

30 surface protein of the target cell. In the case of a cellular receptor, for example, for a cytokine or growth factor, the first binding moiety can be a polypeptide

comprising at least the receptor-binding portion of the
native ligand. A "native ligand" or "native binding
partner" is defined herein as the molecule naturally
produced by, for example, the animal or species which
5   binds to the surface protein in nature. Preferably, the
first binding moiety is a polypeptide or protein. As
such, the native ligand of a cytokine receptor is the
native cytokine. In another embodiment, the first
binding moiety can comprise a binding fragment of an
10   antibody, such as the variable region or a single chain
antibody.

Preferably, the first binding moiety is a poly-
peptide ligand to a cellular receptor. Examples of
preferred ligands are growth factors, epidermal growth
15   factor, interleukins, GM-CSF, G-CSF, M-CSF, EPO, TNF,
interferons, and chemokines. Generally, the cellular
receptor is not presented on the cell which expresses
the viral receptor (e.g., a cell receptor which is bound
by the virus during infection). In one embodiment, the
20   cellular receptor which binds to the first binding
moiety is not an Fc receptor. Thus, in this embodiment,
the bifunctional molecule is not an immunoadhesin.

The first binding moiety can have an amino acid
sequence which is the same or substantially the same as
25   an amino acid sequence of at least the receptor-binding
portion of a native ligand for the cellular receptor.
Similar to cellular receptors, many of the corresponding
ligands have been identified, sequenced and character-
ized, including the portions thereof which bind to the
30   receptor. The first binding moiety can, therefore,
include the same or substantially the same sequence of
the entire native ligand. Alternatively, first binding

moiety comprises the receptor binding portion of the
native ligand, eliminating, in some cases, the effector
function of the ligand.

In another embodiment, the first binding moiety is
5   selected or derived from native ligands or binding
partners to a cellular surface molecule of the target
cell. A "cellular surface molecule" as defined herein
can be a peptide (including post-translationally
modified proteins, such as amidated, demethylated,
10 methylated, prenylated, palmitoylated, glycosylated,
myristylated, acetylated or phosphorylated proteins),
sugar, lipid, steriod, anion or cation, or a combination
thereof which binds the first binding moiety.
Preferably, the binding of the cellular surface molecule
15 to the binding moiety of the bifunctional molecule will
be of high affinity. Examples of high affinity have an
equilibrum constant of 10^{-6} (preferably 10^{-8}) or lower.

The cellular surface molecule need not be
"specific" for the target cell. However, the cellular
20 surface molecule is specific for the desired viral
vector. For example, specific delivery of Influenza A
viral vectors can employ sialic acid cellular surface
molecules for entry into a target cell whereas targeting
of VSV viral vectors can employ a phospholipid as the
25 surface molecule. As such, the cellular surface
molecule for the first binding moiety can be present on
many different cell-types, specific or even unique to
the target cell.

In other embodiments, the effector function can be
30 desirable, thereby stimulating or modulating the
cellular activity of the target cell which can enhance
therapy. An example of where such a therapy can be

-20-

desirable is in the delivery of a negative selection
marker or suicide protein to a tumor where the target
cell is a lymphokine and the ligand is a cytokine.
Where the lymphokine is stimulated, the cell, in
5   addition to delivering the viral vector, can also
possess therapeutic value in the recruitment of an
endogenous immune response against the tumor, thereby
increasing the therapeutic benefit of the therapy.

The phrase "substantially the same sequence" is
10   intended to include sequences which bind the surface
protein and possess a high percentage of (e.g., at least
about 90%, preferably at least about 95%) sequence
identity with the native sequence. The modifications to
the sequence can be conserved or non-conserved, natural
15   and unnatural, amino acids and are preferably outside of
the binding domain. Amino acids of the native sequence
for substitution, deletion, or conservation can be
identified, for example, by a sequence alignment between
proteins from related species or other related proteins.
20   Where the first binding moiety comprises a binding
fragment of an antibody, many antibodies to surface
proteins are known or are commercially available, as are
the amino acid sequences which are responsible for
binding. Alternatively, novel antibodies can be
25   prepared by methods known in the art, such as by Harlow
and Lane, "Antibodies, A Laboratory Manual," Cold Spring
Harbor Laboratory (1988).

Similar to the first binding moiety, the second
binding moiety is a chemical entity which binds to the
30   specified viral target molecule on the viral vector,
generally a surface protein of the viral particle. The
binding can be via a covalent bond, ionic bonding,

-21-

hydrogen bonding or other mechanism. The binding moiety
can be a peptide, sugar, lipid, steroid, nucleic acid,
small molecule, anion or cation, or combination thereof
which binds the specified viral target molecule.

5   Generally, the second binding moiety of the bifunctional
molecule is also a polypeptide. One embodiment of the
second binding moiety comprises an antigen-binding
fragment of an antibody which recognizes and binds to
the viral vector. However, where one binding moiety

10   comprises an antibody fragment, it is preferred that the
other binding molecule does not comprise an antibody
fragment.

     In a more preferred embodiment, the second binding
moiety is a polypeptide which has an amino acid sequence

15   which is the same or substantially the same as an amino
acid sequence of at least the viral-binding portion of a
native extracellular domain of a viral cellular
receptor, such as the viral cellular receptor is on a
cell which is subject to infection by the viral vector.

20   The viral surface protein is thus generally the viral
protein which binds to this cell and activates or
otherwise induces steps for viral entry and, thus,
infection. Where the viral vector is a pseudotyped
viral vector (such as where the vector is a retrovirus

25   containing the envelope protein of another retrovirus,
of an orthomyxovirus or of a rhabdovirus), as discussed
above, it is understood that the viral cellular receptor
generally corresponds to the env protein incorporated
into the viral particle. In the case where the viral

30   surface protein is an env protein, the second binding
partner generally binds the env SU protein and, thereby,
activates the env TM protein, inducing or mediating

-22-

virus-cell membrane fusion and viral entry. As such, a
"viral cellular receptor" is a receptor molecule in or
on a cell which binds to a natural or non-natural viral
particle or protein, for example, in the course of
5   infection. Non-natural viral particles include
pseudotyped viruses, replication defective viruses,
attenuated viruses, or recombinantly engineered viruses.

Examples of viral cellular receptors include the
subgroup A avian leukosis virus receptor, the subgroup B
10   and D avian leukosis virus receptor, the CD4 receptor
for human and simian immunodeficiency viruses, the
amphotropic and ecotropic murine leukemia virus
receptors, the bovine leukemia virus receptor, the
gibbon ape leukemia virus receptor, the poliovirus
15   receptor, the major subgroup rhinovirus receptor (ICAM-
1), the adenovirus and coxsackie virus receptor (CAR),
the coronavirus receptors (e.g. aminopeptidase N and
MHVR), and sialic acid and other carbohydrate receptors
(e.g., for orthomyxoviruses and paramyxoviruses). Many
20   viral cellular receptors are known in the art, including
the sequences thereof.

Cellular receptors are generally transmembrane
proteins comprising intracellular, transmembrane
(characterized by highly hydrophobic regions in the
25   sequence) and extracellular domains. It is generally
preferred that the second binding moiety comprises the
native extracellular domain of the receptor molecule.
As above with the first binding moiety, the second
binding moiety can alternatively comprise the viral
30   binding moiety of the extracellular domain of the
receptor and mutations of the extracellular domain. The
viral binding moiety can be identified or confirmed by

-23-

manufacturing various fragments of the extracellular
domain and screening them for their ability to bind. As
above, viral-binding mutations to the extracellular
domain can be manufactured as well. Mutations to the
5   extracellular domain include conservative and non-
conservative amino acid substitutions, particularly
those not implicated in the binding of the viral
particle. The phrase "substantially the same sequence"
is intended to include sequences which bind the viral
10 protein and possess a high percentage of (e.g., at least
about 90%, preferably at least about 95%) amino acid
sequence identity with the native sequence. The
modifications to the sequence can be conserved or non-
conserved amino acids.

15   The phrase "conservative amino acid substitutions"
is intended to mean amino acids which possess similar
side chains (e.g., hydrophobic, hydrophilic, aromatic,
etc.), as is known in the art.

         The first and second binding moieties can be
20 directly bonded together or through a linking moiety.
Where one or both of the moieties are polypeptides, a
peptide bond or peptide linker is preferred, thereby
obtaining a "fusion protein" of the two moieties which
can be expressed by a single nucleic acid construct in
25 series. The two moieties can alternatively be linked
directly or indirectly other than via a peptide bond or
peptide linker, thereby obtaining a "conjugate".

         Where the moieties are directly bonded to each
other, the bond can be covalent, as in a peptide bond,
30 ionic bonding or hydrogen bonding. Where the bond is a
peptide bond, the first moiety can be bonded to the N-
terminus of the second moiety via the C-terminus, or

vice versa. It is acknowledged that one fusion protein
may possess greater activity than a second fusion
protein due to conformational or steric considerations.

Where one or more of the moieties are not poly-
5   peptides, they can be joined via chemical reaction
through functional groups present on each moiety which,
under the appropriate conditions, will react with each
other. For example, acid groups (or activated
derivatives thereof) can be reacted with amines,
10   alcohols or thiols to form amide or ester bonds, as is
known in the art.

Alternatively and advantageously, a linking moiety
is employed to link the two moieties. The linker can
preferably be a flexible linker and sufficient in length
15   to separate the moieties in space, thereby not
restricting the ability of the bifunctional molecule to
bind independently and maintain the proper conformation.
Again, where both moieties are polypeptides, the linker
moiety will generally be a peptide, polypeptide, or a
20   "pseudopeptide". A "pseudopeptide" is a bifunctional
linker which contains at least one non-amino acid and
reacts to form a peptide bond, or other bond, with the
terminal amine or carboxyl group of the moiety. For
example, a peptide characterized by substitution of the
25   terminal amine for a carboxyl group can function to
react with the amine terminus of each moiety. Such as
linker is considered to be a "pseudopeptide." Similarly,
a peptide characterized by substitution of the terminal
carboxyl for an amine group can function to react with
30   the carboxyl terminus of each moiety.

Generally, however, the linker will be a peptide
linker which will link the amine terminus of one moiety

-25-

to the carboxyl terminus of the second moiety. One
advantage to such a molecule is the ability to express
the bifunctional molecule as a fusion protein in a
recombinant host cell with a single nucleic acid

5   construct.

Preferred peptide linkers can be obtained from
immunoglobulin hinge regions, such as a proline rich
region. Also, linkers can be characterized by little
steric hindrance, thereby permitting maximal independent

10   movement of the two moieties, such as with a polyglycine
linker. Alternatively, the linker selected to be
reactive to or inert to cellular proteases can be
desirable. In another embodiment, the linker can be
selected to avoid or minimize an immune response against

15   the fusion protein. The length of the linker also is
not particularly critical. Typically, the length of the
linker will be between about 2 and about 20 amino acids.
As can be seen, the selection of the particular linking
group is not critical to the invention.

20   In yet another embodiment, the linker can be a
bifunctional compound which will react with other
functional groups on the two binding moieties, such as
in the reaction of acids and amines or alcohols (as
present in peptides, carbohydrates and lipids, for

25   example) in the formation of amides or esters.

A preferred combination of the above first and
second binding moieties includes the selection of a
polypeptide ligand to a cell-type specific cellular
receptor linked, via a peptide linker through a terminus

30   of the ligand to the terminus of at least the viral-
binding moiety of the extracellular domain of a viral

cellular receptor (or a mutant thereof, as defined above).

For example, the C-terminus of the first binding polypeptide is linked to the N-terminus of the second
5   binding polypeptide via the polypeptide linker or the N-terminus of the first binding polypeptide is linked to the C-terminus of the second binding polypeptide via the polypeptide linker.

A particularly preferred combination of these
10 elements is for targeting a retroviral vector to a cell-type comprises a receptor-binding fragment of a polypeptide ligand which binds a cellular receptor specific to the desired cell-type and a viral-binding fragment of a cellular receptor for the retroviral
15 vector. For example, the polypeptide ligand can be human epidermal growth factor. Where the retroviral vector is avian leukosis virus, the cellular receptor can be Tva. The two polypeptides can then be linked via a hinge region, such as a proline-rich hinge region.

20   In another aspect of the invention, peptidomimetics (molecules which are not polypeptides, but which mimic aspects of their structures to bind to the same site), that are based upon the above-described polypeptides, can also be used as binding moieties of the bifunctional
25 molecules. For example, polysaccharides can be prepared that have the same functional groups as the polypeptide binding moieties of the invention, and which interact with target cell and viral surface molecules in a similar manner. Peptidomimetics can be designed, for
30 example, by establishing the three dimensional structure of the polypeptide in the environment in which it is bound or will bind to the target cell or viral vector.

The peptidomimetic comprises at least two components, a binding entity or entities and a backbone or supporting structure entity.

The binding entities of the peptidomimetic are the
5   chemical atoms or groups which will react or complex (as in the formation of a hydrogen or covalent bond) with the target cell or viral surface molecule. In general, the binding entities in a peptidomimetic are the same as the polypeptide of the bifunctional molecule.
10   Alternatively, the binding entities can be an atom or chemical group which will react with the target cell or viral surface molecule in the same or similar manner as the polypeptide. Examples of binding entities suitable for use in designing a peptidomimetic for a basic amino
15   acid in a polypeptide are nitrogen containing groups, such as amines, ammoniums, guanidines and amides or phosphoniums. Examples of binding entities suitable for use in designing a peptidomimetic for an acidic amino acid in a polypeptide can be, for example, carboxyl,
20   lower alkyl carboxylic acid ester, sulfonic acid, a lower alkyl sulfonic acid ester or a phosphorous acid or ester thereof.

The supporting structure is the chemical entity that, when bound to the binding moiety or moieties,
25   provides the three dimensional configuration of the peptidomimetic. The supporting structure can be organic or inorganic. Examples of organic supporting structures include polysaccharides, polymers (such as, polyvinyl alcohol or polylactide). It is preferred that the
30   supporting structure possess substantially the same size and dimensions as the polypeptide backbone or supporting structure. This can be determined by calculating or

-28-

measuring the size of the atoms and bonds of the
polypeptide and peptidomimetic. For example, the
nitrogen of the peptide bond can be substituted with
oxygen or sulfur, thereby forming a polyester backbone.

5   Likewise, the carbonyl of the peptide bond can be
substituted with a sulfonyl group or sulfonyl group,
thereby forming a polyamide. Reverse amides of the
peptide can be made (e.g., substituting one or more
-CONH- groups for a -NHCO- group). In addition, the

10 peptide backbone can be substituted with a polysilane
backbone.

    These peptidomimetic compounds can be manufactured
by art-known and art-recognized methods. For example, a
polyester corresponding to the peptide RRKRK can be

15 prepared by the substituting a hydroxyl group for each
corresponding amine group on the R and K amino acids,
thereby preparing a hydroxyacid and sequentially
esterifying the hydroxyacids, optionally blocking the
basic side chains and acids to minimize side reactions.

20 Determining an appropriate chemical synthesis route can
generally be readily identified upon determining the
chemical structure using no more than routine skill.

    Also encompassed by the present invention are the
use of bifunctional molecules for detecting ligand and

25 ligand-cell specific receptor binding pairs. The ligand
and/or receptor can be peptides (including post-
translationally modified proteins) and/or small
molecules (including sugars, steroids, lipids, anions or
cations). The ligands and ligand-cell specific

30 receptors can be known or unknown. Where the ligand is
known and the receptor is unknown, ligand-cell specific
receptors can be identified, for example, by screening

host cells transfected with nucleotides encoding
potential receptors. For example, the ligands can be
secreted (such as chemokines) or non-secreted (such as
the extracellular domains of chemokines receptors)
5   proteins.

A library of host cells displaying putative ligand-
cell surface receptors can be obtained by transfecting
suitable host cells with nucleic acid constructs,
including but not limited to cDNA or genomic libraries,
10 under appropriate regulatory control to result in the
expression of cell-surface receptors on the host cell.
The bifunctional molecule with ligand and an appropriate
viral vector, e.g., containing an inducible reporter
gene (such as β-galactosidase or chloramphenicol acetyl
15. transferase), is added to the population of host cells
under conditions suitable for viral infection.
Following infection, infection can be detected. For
example, reporter gene expression can be induced under
appropriate conditions and the host cells which express
20 the ligand receptor can be identified. Experimental
conditions for viral vector expression and induction and
detection of reporter genes are well known in the art.
A similar approach can be used to select unknown ligands
in the case where the ligand-cell specific receptors are
25 known and the ligand is unknown. In this embodiment, a
library of bifunctional molecules with putative ligands
(e.g., chemokines) can be obtained and contacted with
one or more host cells displaying cell surface receptors
and an appropriate viral vector. Infection can be
30 detected as described above.

Thus, the invention relates to a method for
detecting ligand-receptor binding pairs comprising

contacting, under conditions suitable for infection, a
mixture comprising a viral vector, a bifunctional
molecule and a host cell, wherein the bifunctional
molecule comprises a ligand and a binding moiety which
5   binds to a viral vector surface molecule on the viral
vector and activates viral entry into a host cell
expressing a receptor. Ligand binding to a receptor can
be indicated by viral vector infection of the host cell
by, for example, the use of reporter genes. The host
10 cells can display receptors distinct for the known
ligand or can express recombinant receptors comprising a
collection of nucleic acid constructs encoding ligand-
cell surface receptors.

        A similar approach can be used to identify unknown
15 ligands wherein the cell surface receptor is known,
where the bifunctional molecule comprises a receptor and
a binding moiety which binds a viral surface molecule.
The host cell expresses a distinct ligand or a
collection of recombinant ligands. Ligand-receptor
20 binding can be detected following infection of the viral
vector to the host cell.

        The bifunctional molecules can be manufactured
according to methods generally known in the art. For
example, where one or both of the binding moieties is a
25 nonpeptide the bifunctional molecule can be manufactured
employing known organic synthesis methods useful for
reacting a functional or reactive group on the moiety
with a functional or reactive group on the other moiety
or, preferably, a linker. In carrying out the
30 synthesis, derivation or inactivation of the functional
group(s) required for binding to the moiety's binding
partner should be avoided. Appropriate syntheses are

-31-

highly dependent upon the chemical nature of the binding
moiety and, generally, can be selected from an advanced
organic chemistry text, such as March, *et al.* *Advanced
Organic Chemistry*, 3rd Edition (1985) John E. Wiley &
5   Sons, Inc., New York, NY, or other known methods.

     Where the binding moieties are polypeptides, the
bifunctional molecule can be a conjugate or a fusion
protein and manufactured according to known methods.
Where a fusion protein is desired, the molecule can be
10   manufactured according to known methods of recombinant
DNA technology. For example, the fusion protein can be
expressed by a nucleic acid molecule comprising
sequences which code for both moieties, such as by a
fusion gene. Thus, the invention further relates to
15   nucleic acid molecules which encode the bifunctional
molecule.

     The nucleic acid molecule can be double stranded or
single stranded and can be a DNA molecule, such as cDNA
or genomic DNA, or an RNA molecule. As such, the
20   nucleic acid molecule can include one or more exons,
with or without, as appropriate, introns. In one
example, the nucleic acid molecule contains a single
open reading frame which encodes both the first binding
moiety, second binding moiety and optionally a signal
25   sequence and/or a polypeptide linker, when present. By
way of example in a multi-exon construct, the nucleic
acid molecule contains a first exon which begins with an
ATG, encodes one of the binding partners, and optionally
the polypeptide linker, and ends with a splice donor
30   site. The construct further would contain an intron
followed by a second exon which begins with a splice
acceptor site and, optionally a polypeptide linker,

-32-

coding sequences for the other binding partner and
ending with a stop codon. Of course, alternative
combinations of these elements would be apparent to the
person of skill in the art.

5   As such, the nucleic acid molecule can include
sequences which encode the first and second binding
partners, as well as one or more of the following
optional sequences, in a functional relationship,
regulatory sequences (as will be discussed in more

10   detail below) a start codon, a signal or leader
sequence, splice donor sites, splice acceptor sites,
introns, a stop codon, transcription termination
sequences, 5' and 3' untranslated regions, polyadenyl-
ation sequences, negative and/or positive selective

15   markers, and replication sequences.

          The coding regions of the nucleic acid molecule
code for the first and second binding moieties of the
bifunctional molecule and any polypeptide linkers
present. Where the binding moiety is a native ligand or

20   cellular surface protein (e.g. a cellular receptor), or
a binding fragment thereof, the nucleic acid molecule
coding regions can correspond to the native sequences
which encode the binding moiety. Because many amino
acids are encoded by a plurality of codons, the coding

25   sequence can be mutated to result in the same amino acid
sequence. This may be advantageous where a codon is
preferred by the selected host cell. Where the binding
moiety is a mutation or variant of a native sequence, as
provided above, generally, the nucleic acid sequence

30   will be mutated correspondingly. It is preferred for
ease of manufacture of the nucleic acid

sequence to maintain as much of the native sequence as
possible. In one embodiment, the nucleic acid molecule
shares at least about 65% sequence identity with the
corresponding native sequence, preferably, at least
5   about 75% sequence. In a more preferred embodiment, the
percent sequence identity is at least about 85%, and
still more preferably, at least about 95%.

Recombinant nucleic acid molecules meeting these
criteria comprise nucleic acids having sequences
10 identical to sequences of naturally occurring genes,
including polymorphic or allelic variants, and portions
(fragments) thereof, or variants of the naturally
occurring genes. Such variants include mutants
differing by the addition, deletion or substitution of
15 one or more residues, modified nucleic acids in which
one or more residues are modified (e.g., DNA or RNA
analogs), and mutants comprising one or more modified
residues.

Many nucleic acid molecules coding for suitable
20 binding moieties are known in the art and can be
obtained from, for example, GENBANK. Alternatively,
other sequences can be employed, such as homologs of
known genes.

Such homologous nucleic acids, including DNA or
25 RNA, can be detected and/or isolated by hybridization
(e.g., under high stringency conditions or moderate
stringency conditions). "Stringency conditions" for
hybridization is a term of art which refers to the
conditions of temperature and buffer concentration which
30 permit hybridization of a particular nucleic acid to a
second nucleic acid in which the first nucleic acid may
be perfectly complementary to the second, or the first

and second may share some degree of complementarity
which is less than perfect. For example, certain high
stringency conditions can be used which distinguish
perfectly complementary nucleic acids from those of less
5 complementarity. "High stringency conditions" and
"moderate stringency conditions" for nucleic acid
hybridizations are explained on pages 2.10.1-2.10.16
(see particularly 2.10.8-11) and pages 6.3.1-6 in
Current Protocols in Molecular Biology (Ausubel, F.M. *et
10 al*., eds., Vol. 1, containing supplements up through
Supplement 29, 1995), the teachings of which are hereby
incorporated by reference. The exact conditions which
determine the stringency of hybridization depend not
only on ionic strength, temperature and the
15 concentration of destabilizing agents such as formamide,
but also on factors such as the length of the nucleic
acid sequence, base composition, percent mismatch
between hybridizing sequences and the frequency of
occurrence of subsets of that sequence within other non-
20 identical sequences. Thus, high or moderate stringency
conditions can be determined empirically.

By varying hybridization conditions from a level of
stringency at which no hybridization occurs to a level
at which hybridization is first observed, conditions
25 which will allow a given sequence to hybridize (e.g.
selectively) with the most similar sequences in the
sample can be determined.

Exemplary conditions are described in Krause, M.H.
and S.A. Aaronson, *Methods in Enzymology*, 200:546-556
30 (1991). Also, see especially page 2.10.11 in *Current
Protocols in Molecular Biology (supra)*, which describes
how to determine washing conditions for moderate or low

stringency conditions. Washing is the step in which
conditions are usually set so as to determine a minimum
level of complementarily of the hybrids. Generally,
starting from the lowest temperature at which only
5 homologous hybridization occurs, each °C by which the
final wash temperature is reduced (holding SSC
concentration constant) allows an increase by 1% in the
maximum extent of mismatching among the sequences that
hybridize. Generally, doubling the concentration of SSC
10 results in an increase in T_m of ~17°C. Using these
guidelines, the washing temperature can be determined
empirically for high, moderate or low stringency,
depending on the level of mismatch sought.

The nucleic acid molecule also preferably comprises
15 regulatory sequences. Regulatory sequences include all
cis-acting elements that control transcription and
regulation such as, promoter sequences, enhancers,
ribosomal binding sites, and transcription binding
sites. Selection of the promoter will generally depend
20 upon the desired route for expressing the protein. For
example, where the protein will be transformed into a
cell by a viral vector, preferred promoter sequences
include viral, such as retroviral or adenoviral
promoters. Examples of suitable promoters include the
25 cytomegalovirus immediate-early promoter, the retroviral
LTR, SV40, and TK promoter. Where the protein is to be
expressed in a recombinant eukaryotic or prokaryotic
cell, the selected promoter is recognized by the host
cell. A suitable promoter which can be used can include
30 the native promoter for the binding moiety which appears
first in the construct.

The elements which comprise the nucleic acid molecule can be isolated from nature, modified from native sequences or manufactured *de novo*, as described, for example, in the above-referenced texts. The

5 elements can then be isolated and fused together by methods known in the art, such as exploiting and manufacturing compatible cloning or restriction sites.

The nucleic acid molecules can be inserted into a construct which can, optionally, replicate and/or

10 integrate into a recombinant host cell, by known methods. The host cell can be a eukaryotic or prokaryotic cell and includes, for example, baculo-viruses, pichia expression systems, yeast (such as Saccharomyces), bacteria (such as, Escherichia or

15 Bacillus), animal cells or tissue, including insect or mammalian cells (such as, somatic or embryonic human cells, Chinese hamster ovary cells, HeLa cells, human 293 cells and monkey COS-7 cells, etc.).

The nucleic acid molecule can be incorporated or

20 inserted into the host cell, also, by known methods. Examples of suitable methods of transfecting or transforming cells include calcium phosphate precipitation, electroporation, microinjection, infection, lipofection and direct uptake. Methods for preparing

25 such recombinant host cells are described in more detail in Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual," Second Edition (1989) and Ausubel, *et al.* "Current Protocols in Molecular Biology," (1992), for example.

30 The host cell is then maintained under suitable conditions for expression and recovering the bifunctional molecule. Generally, the cells are

-37-

maintained in a suitable buffer and/or growth medium or
nutrient source for growth of the cells and expression
of the gene product(s). The growth media are not
critical to the invention, are generally known in the
5   art and include sources of carbon, nitrogen and sulfur.
Examples include Dulbeccos modified eagles media (DMEM),
RPMI-1640, M199 and Grace's insect media. Again, the
selection of a buffer is not critical to the invention.
The pH which can be selected is generally one tolerated
10 by or optimal for growth for the host cell.

The cell is maintained under a suitable temperature
and atmosphere. Anaerobic host cells are generally
maintained under anaerobic conditions. Alternatively,
the host cell is aerobic and the host cell is maintained
15 under atmospheric conditions or other suitable
conditions for growth. The temperature should also be
selected so that the host cell tolerates the process and
can be for example, between about 35° and 40°C.

The bifunctional molecule produced by the processes
20 described herein can be isolated and purified by known
means. Examples of suitable purification and isolation
processes are generally known and include ammonium
sulfate precipitation, dialysis, gel filtration, immuno-
affinity, chromatography, electrophoresis, ultrafiltra-
25 tion, microfiltration or diafiltration.

The bifunctional molecule is preferably purified
substantially prior to use, particularly where the
protein will be employed as an *in vivo* therapeutic,
although the degree of purity is not necessarily
30 critical where the molecule is to be used *in vitro*. In
one embodiment, the bifunctional molecule can be
isolated to about 50% purity (by weight), more

preferably to about 80% by weight or about 95% by weight. It is most preferred to employ a molecule which is essentially pure (e.g., about 99% by weight or to homogeneity).

5   Bifunctional molecules which are prepared according to the above method can be used directly in the disclosed method or can be screened for activity prior to use. To screen the bifunctional molecule for activity, for example, *in vitro*, the bifunctional

10 molecule (or mixtures of bifunctional molecules) can be contacted with, for example, the target cell or the targeted surface protein under conditions suitable for binding and then assayed for binding. Similarly the bifunctional molecule can be screened for the ability to

15 bind the viral vector, or the surface protein of the viral vector, *in vitro*, by contacting the vector and bifunctional molecule under conditions suitable for binding and detecting binding. The bifunctional molecule can be screened for the ability to induce viral

20 entry by contacting the vector, bifunctional molecule and target cell under typical cell culture conditions and detecting infection of the cells (e.g. replication of viral sequences).

     The bifunctional molecule of the invention is

25 particularly useful in the delivery of one or more polynucleotides (e.g., genes) or products thereof to a patient. Generally, the polynucleotide is present or has been incorporated into the genome of the viral vector. The polynucleotide or the product thereof can

30 be a therapeutic agent. An example of a therapeutic polynucleotide includes RNA (e.g., ribozymes) and antisense DNA that prevents or interferes with the

-39-

expression of an undesired protein in the target cell.
The polynucleotide can also encode a heterologous
therapeutic protein. A heterologous protein or
polynucleotide is one which does not exist in the virus
5   as it is found in nature. Examples of therapeutic
proteins include antigens or immunogens such as a
polyvalent vaccine, cytokines, tumor necrosis factor,
interferons, interleukins, adenosine deaminase, insulin,
T-cell receptors, soluble CD4, epidermal growth factor,
10 human growth factor, blood factors, such as Factor VIII,
Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC,
ApoAI, the LDL receptor, negative selection markers or
"suicide proteins", such as thymidine kinase (including
the HSV, CMV, VZV TK), anti-angiogenic factors, Fc
15 receptors, plasminogen activators, such as t-PA, u-PA
and streptokinase, dopamine, MHC, tumor suppressor genes
such as p53 and Rb, monoclonal antibodies or antigen
binding fragments thereof, drug resistance genes, ion
channels, such as a calcium channel or a potassium
20 channel, and adrenergic receptors, etc.
        The invention is particularly useful for vaccine
delivery. In this embodiment, the antigen or immunogen
can be expressed heterologously (e.g., by recombinant
insertion of a nucleic acid sequence which encodes the
25 antigen or immunogen (including antigenic or immunogenic
fragments) in a viral vector). Alternatively, the
antigen or immunogen can be expressed in a live
attenuated, pseudotyped virus vaccine, for example.
Generally, the methods can be used to generate humoral
30 and cellular immune responses, e.g. via expression of
heterologous pathogen-derived proteins or fragments
thereof in specific target cells.

-40-

Generally, viral vectors which contain therapeutic polynucleotide are known in the art. Examples include the vectors described in Anderson, *et al.* (United States Patent No.5,399,346), Sambrook, *et al.*, *supra*, Ausubel,

5   *et al.*, *supra*, and Weiss, *et al.* (1985) "RNA Tumor Viruses", Cold Spring Harbor, NY, the contents of which are incorporated herein by reference.

The bifunctional molecule of the claimed invention can be employed in a method for delivering a viral

10   vector to a cell comprising contacting the cell with the bifunctional molecule. The cell can be contacted with the viral vector and the bifunctional molecule *in vitro* or *in vivo*. Where the cell is contacted *in vitro*, the target cell containing the viral vector can then be

15   implanted into a patient for delivery of the poly-nucleotide or product thereof. The target cell can be migratory, such as a hematopoietic cell, or non-migratory, such as a solid tumor cell or fibroblast. Frequently, the target cell is targeted from the patient

20   and returned to the patient once contacted with the bifunctional molecule and viral vector. *Ex vivo* gene therapy has been described, for example, in Kasid, *et al. PNAS USA* 87:473 (1990), Rosenberg *et al. N. Engl. J. Med.*, *323:* 570 (1990), Williams, *et al.*, *Nature* 310:476

25   (1984), Dick, *et al.*, *Cell* 42:71 (1985), Keller, *et al. Nature 318*:149 (1985) and Anderson, *et al.* United States Patent No. 5,399,346.

Alternatively, the bifunctional molecule can be coadministered to the patient with the viral vector.

30   Coadministration is meant to include simultaneous or sequential administration of the two agents, individually or in combination. Where the agents are

administered individually it is preferred that the mode
of administration is the same and the site of admini-
stration is the same to improve efficiency. It is also
preferred that the administrations are conducted

5 sufficiently close in time so that the active
ingredients are in the presence of the cells
simultaneously. In one embodiment, the bifunctional
molecule is administered prior to the viral vector so
that the molecule can bind to the target cell initially.

10 In another embodiment, the bifunctional molecule is
contacted with the viral vector prior to administration
so that the vector and molecule can bind prior to
contact with the target cell.

The mode of administration is preferably at the

15 location of the target cells. As such, the
administration can be nasally (as in administering a
vector expressing ADA) or by injection (as in
administering a vector expressing a suicide gene to a
tumor). Other modes of administration (parenteral,

20 mucosal, systemic, implant, intraperitoneal, etc.) are
generally known in the art. The agents can, preferably,
be administered in a pharmaceutically acceptable
carrier, such as saline, sterile water, Ringer's
solution, and isotonic sodium chloride solution.

25 The examples presented below are provided as
further guidance and are not to be construed as limiting
the invention in any way.

-42-

Exemplification

EXAMPLE 1

A modified form of a human EGF minigene was
obtained by PCR amplification of AX proviral DNA using
5   the oSS1 and oSS2 primers described below. The modified
form of the human EGF minigene encoded human EGF (Stern,
1987, *Science 235*:322) with a polyproline linker region
(PPPELLGGP) derived from a rabbit Fc chain, added at the
N-terminal end. The PCR product contained an EagI site
10 introduced at the 5' end, and KpnI and EagI sites
following the human EGF stop codon. The PCR product was
digested with the EagI restriction enzyme and introduced
into the EagI site of a synthetic Tva gene (Belanger,
1996, *J. Virol. 69*:1019-1024) contained within the pCI
15 mammalian expression vector (Promega).

oSS1:   gcgcggccgcccacccctgaactcctgggggggaccggaggttcag
           aactctgattccgaatgc (SEQ ID NO:1)

oSS2:   gcgcggccgggtaccttatcgcagttccaatttcaggtcgcg
           (SEQ ID NO:2)

20   Human 293 cells grown under standard conditions
(Zingler, *J. Virol.* 70:7510-7516 (1996)) were plated at
20% confluence on 100 mm tissue culture plates. The
cells were transfected with 15μg of the plasmid
expression vector encoding TVA-EGF (described above) by
25 the calcium phosphate transfection procedure (Wigler
Cell 11:223-232 (1977)). Radioactively-labeled TVA-EGF
was prepared by incubating transfected 293 cells with ^{35}S
cysteine: forty eight hours after transfection, the
transfected cells (or for control purposes,

-43-

untransfected cells) were incubated with cysteine/
methionine-free DME (1% dialyzed calf serum) for 1 hour
and then with the same media supplemented with 100µCi/ml
35S-cysteine for 2 hours. The cells were then washed
5   with PBS and incubated with DMEM for 4 hours and the
extracellular supernatants were harvested and stored at
-80°C before analysis. Aliquots of 500µl labeled cell
culture supernatants were incubated with either 12µg of
a subgroup A SU-immunoadhesin (SUA-rIgG)(Zingler, J.
10 Virol. 70:7510-7516 (1996)) or with 26 µg of an antibody
specific for human EGF for 1 hour at 4°C. These samples
were then incubated with protein A-Sepharose, or protein
G-Sepharose, respectively for 1 hour at 4°C. The
precipitated proteins were then collected by
15 centrifugation in a microfuge, the sepharose beads were
washed 4 times each with 1 ml of ice cold PBS and the
samples boiled in a non-reducing protein sample buffer
for 3 minutes. The proteins were then subjected to
electrophoresis on a 10% polyacrylamide gel containing
20 SDS, the gel was then dried and exposed to Kodak XAR-5
film at room temperature. Alternatively, extracellular
supernatants were collected 72 hours after transfection
and 45 µl aliquots subjected to electrophoresis on 10%
polyacrylamide gels containing SDS under non-reducing
25 conditions. Electrophoretically separated proteins were
then transferred to nitrocellulose and the TVA-EGF
detected by immunoblotting with SUA-rIgG followed by
horseradish peroxidase (HRP)-conjugated secondary
antibody specific for rabbit immunoglobulins and
30 enhanced chemiluminescence (Zingler, et al., J. Virol.
70:7510 (1996)). Transiently transfected human 293
cells produced bifunctional molecules containing TVA and

-44-

EGF (TVA-EGF) in the extracellular supernatant whereas
supernatants from untransfected control cells did not
produce TVA-EGF.

EXAMPLE 2

5   Unlabeled TVA-EGF was collected in extracellular
supernatants from 293 cells transfected essentially as
described above except that the supernatants were
harvested 72 hours post-transfection. These
supernatants were filtered through a 0.45 μm filter and

10   stored at -20°C before use. Confluent plates of cells
were washed with Ca^{2+}/Mg^{2+} free PBS and the cells were
removed from the plates with 5 ml of Ca^{2+}/Mg^{2+} free PBS
containing 1 mM EDTA and put on ice. Mouse L cells
lacking EGF receptors (B82), expressing wild type EGF

15   receptors (T23) or expressing EGF receptors lacking in
kinase activity (M5) (Chen, 1987, *Nature 328*:820) (4 x
10^3 cells) were incubated either with unlabeled TVA-EGF
supernatant (0.1, 0.5, 2.5, 12.5, 25, 250 or 500 μl) or
with untransfected 293 cell supernatant for 30 minutes

20   on ice. The cells were spun in a microfuge and washed
with ice cold PBS and incubated with 1 μg of purified
SUA-rIgG or in 1 ml of PBS for 30 minutes on ice. After
washing the cells once with ice cold PBS, the cells were
incubated with 5 μl of FITC-conjugated swine anti-rabbit

25   antibody (DAKO) for 30 minutes on ice. Finally, the
cells were washed once with ice cold PBS and resuspended
in 0.5 ml of PBS containing 1% formaldehyde. The cells
were kept in the dark at 4°C before analysis. Bound
proteins were detected by flow cytometry using a Becton

30   Dickinson FACScan. The TVA-EGF bound subgroups A SU-
immunoadhesin to the M5 and T23 cells that express

-45-

surface EGF receptors (M5 and T23 cells), but not to the
B82 cells which lack the EGF receptor (Figure 2). The
amount of TVA-EGF bound to cells, as measured by mean
fluorescence, increased with increasing amounts of TVA-
5   EGF up to concentrations of 12.5 μg. Amounts of TVA-EGF
higher than 12.5 μg did not result in a significant
increase in the amount of TVA-EGF bound to cells. Thus,
the TVA-EGF bifunctional molecule is capable of dual
binding as indicated by binding to both an EGF receptor
10 on the surface of M5 and T23 target cells and the SUA-
rIgG antibody-like protein.

    EXAMPLE 3
        The specificity of TVA-EGF binding was evaluated by
15 competition experiments using recombinant human EGF
    (Upstate Biotechnology).
        Bifunctional molecules containing TVA and EGF (TVA-
    EGF) produced in the extracellular supernatant from
    human 293 cells were collected 72 hours post-
20 transfection as described in Examples 1 and 2. M5 cells
    were plated and incubated at 4°C for 1 hour with 200 μl
    of extracellular supernatants containing TVA-EGF as
    described in Example 2 except that varying amounts of
    recombinant human EGF (0-5 μg) were added to the
25 incubation media. The amount of TVA-EGF bound was
    determined by flow cytometry detection using SUA-rIgG as
    described in Example 2. The amount of TVA-EGF bound to
    M5 cells, as measured by mean fluorescence, decreased
    with increasing amounts of recombinant EGF (Figure 3).
30 These data show that the binding of TVA-EGF to EGF
    receptors was specific for the EGF moiety of the TVA-EGF
    bifunctional molecule. As a control, the extracellular

-46-

supernatants of T23TVA cells, which stably express a
transmembrane form of TVA, were also incubated with
increasing amounts of recombinant EGF. T23TVA cells
were generated using previously described procedures

5   (Zingler et al., J. Virol. 69:4261 (1995)). EGF did not
inhibit SUArIgG-TVA binding to T23TVA cells.

EXAMPLE 4

        As further evidence that the TVA-EGF bifunctional
molecule binds to the surface of target cells through

10   EGF receptors, the retention of TVA-EGF proteins on the
surface of M5 and T23 cells was determined.

        M5 and T23 cells were prepared and incubated with
200 μl of extracellular supernatant containing TVA-EGF
(4°C, 1 hour) as described in Example 2. Cells were then

15   washed with ice cold PBS and either left on ice (zero
time point) or incubated with 500 μl of Dulbecco's
Modified Eagles Medium (DMEM) at 37°C conditions for 30,
60 or 120 minutes. Following incubation in DMEM, cells
were placed on ice and the presence of the TVA-EGF

20   bifunctional molecule on the cell surface of M5 and T23
cells detected by flow cytometry as described in Example
2. TVA-EGF remained present at the cell surface of M5
cells, which express EGF receptors lacking kinase
activity for a longer period of time than to T23 cells,

25   which express wildtype EGF receptors (Figure 4). The
majority of TVA-EGF present at the cell surface of T23
cells was cleared from the surface of cells within 30
minutes whereas a substantial amount of TVA-EGF remained
on the surface of M5 cells after 120 minutes. These

30   data and the data presented in Figure 3 indicate that
binding of TVA-EGF to target cells occurs by the EGF

-47-

receptor. Those cells which are deficient in kinase
activity, the M5 cells, retain bound TVA-EGF on their
surface longer than wildtype T23 cells.

EXAMPLE 5

5   M5 cells expressing kinase-deficient EGF receptors
were plated at 10% confluence on 60 mm tissue culture
plates in DME containing 10% dialyzed bovine calf serum
for one day. The plates were then placed on ice, washed
with ice cold PBS, and incubated with 1 ml of either ice
10   cold untransfected 293 cell culture supernatant or with
1 ml ice cold supernatant containing TVA-EGF for 1 hour
at 4°C. The plates were put on ice and washed with ice
cold PBS and then incubated with 1 ml of untransfected
293 cell culture supernatant containing or lacking
15   300 μl of RCASH-A (a subgroup A-specific virus)
containing the hygromycin B-phosphotransferase gene
(Young, 1993, *J. Virol.* 67:1811) (titer 8 x 10^3 IU/ml).
After 1 hour of incubation at 4°C, the plates were
placed in a 37°C incubator and 24 hours later they were
20   selected in medium containing sufficient amounts of
hygromycin B to select against growth of any uninfected
cells (700 μg for M5 cells and 900 μg for B82 cells).
After two weeks of selection, the cells were fixed in
the solution containing 1% (w/v) methylene blue, 20%
25   (v/v) iso-propanol and 5% (v/v) acetic acid and the
colonies were counted. Cells incubated with TVA-EGF
became susceptible to subgroup A viral infection
(Figures 5 and 6). Only M5 cells incubated with
supernatants containing TVA-EGF were infected (Figures 5
30   and 6). TVA-EGF rendered M5 cells highly susceptible
to viral infection. The average number of M5 colonies

-48-

infected with RCASH-A virus was approximately 23% of
that observed after viral infection of T23TVA cells
which express a fully functional epitope-tagged TVA
protein (Figure 6). B82 cells which do not express EGF
5   receptors were not infected following the addition of
TVA-EGF.

EXAMPLE 6

T23 cells, which express the wildtype EGF; M5
10   cells, which express an EGF receptor lacking kinase
activity; or B82 cells, which lack the EGF receptor,
were plated and incubated with 400 μl of subgroup A ALV
virus containing a neomycin phosphotransferase gene
(RCASA-Neo) at 37°C for 28 hours as described in Example
15   5 except that varying amounts of extracellular
supernatant containing TVA-EGF were added (0, 0.5, 2.5,
12.5 or 200 μl). T23TVA and B82 cells were run in
parallel in infectivity experiments as a positive and
negative controls, respectively. T23TVA cells (positive
20   control) were plated at 4°C for 1 hour, incubated with
1.5 ml of DMEM with or without 50 μl of the RCASA-Neo
virus followed by incubation at 37°C for 28 hours before
selection in medium containing 200 μg/ml Geneticin
(G418). After two weeks of selection, infected cells
25   (G418- resistant colonies) were determined as described
in Example 5. Infectivity increased in M5 and T23 cells
with increasing amounts of TVA-EGF in the incubation
media (Figure 7). The average number of infected cells
observed when M5 cells were incubated with an excess
30   amount of TVA-EGF (200 μl) was approximately 10% of that
obtained with T23TVA cells. The level of infectivity

-49-

obtained with T23 cells was lower than that observed with M5 cells (Figure 7).

EXAMPLE 7

Infectivity of M5 cells was diminished by the
5   addition of recombinant human EGF (0.5 and 5 µg) (Figure
8).   In these experiments M5 cells (1 X 10⁵) were plated
on 60 mm plates and incubated with 1 ml ice cold DMEM
containing 200 µl of TVA-EGF extracellular supernatant
and either 0, 0.5 or 5 µg human recombinant EGF.   T23TVA
10 cells were incubated with 1 ml DMEM and either 0 or 5 µg
EGF.   After washing in ice cold PBS the cells, 1.5 ml of
DMEM containing 150 µl of RCASA-Neo was added and the
cells were incubated at 37°C for 28 hours.   The number of
infected G418 resistant colonies was determined as
15 described in Example 6.   Thus, TVA-EGF mediated viral
infection of target cells expressing EGF occurs by
binding of the EGF moiety to EGF cell surface receptors
(Figure 9).

<u>EQUIVALENTS</u>

20   Those skilled in the art will know, or be able to
ascertain, using no more than routine experimentation,
many equivalents to the specific embodiments of the
invention described herein.   These and all other
equivalents are intended to be encompassed by the
25 following claims.

CLAIMS

What is claimed is:

1. A bifunctional molecule comprising:
    (a) a first binding moiety which binds to a first
         cellular surface molecule on a target cell;
         and
    (b) a second binding moiety which binds to a viral
         surface molecule on a viral vector and
         activates viral entry, wherein said second
         binding moiety is an extracellular domain of a
         viral cellular receptor or a derivative
         thereof and said bifunctional molecule is not
         an immunoadhesin.

2. The bifunctional molecule of Claim 1 wherein the
    first cell surface molecule is selected from a
    group consisting of carbohydrates, lipids,
    polypeptides, and glycosylated polypeptides.

3. The bifunctional molecule of Claim 1 wherein the
    first binding moiety is a ligand to a cellular
    receptor.

4. The bifunctional molecule of Claim 3 wherein the
    cellular receptor is cell-type specific.

5. The bifunctional molecule of Claim 4 wherein the
    first binding moiety is a polypeptide.

6. The bifunctional molecule of Claim 5 wherein the ligand has an amino acid sequence which is the same or substantially the same as an amino acid sequence of at least the receptor-binding portion of a native ligand for the cellular receptor.

7. The bifunctional molecule of Claim 5 wherein the ligand has an amino acid sequence which is the same as an amino acid sequence of at least the receptor-binding portion of a native ligand for the cellular receptor.

8. The bifunctional molecule of Claim 5 wherein the target cell is selected from the group consisting of epithelial cells, fibroblasts, smooth muscle cells, blood cells, tumor cells, cardiac muscle cells, macrophages, dendritic cells, neuronal cells, and pathogen-infected cells.

9. The bifunctional molecule of Claim 5 wherein the cellular receptor is selected from the group consisting of the subgroup A avian leukosis virus receptor, the subgroup B and D avian leukosis virus receptor, the CD4 receptor for human and simian immunodeficiency viruses, the amphotropic and ecotropic murine leukemia virus receptors, the bovine leukemia virus receptor, the gibbon ape leukemia virus receptor, the poliovirus receptor, the major subgroup rhinovirus receptor, the adenovirus and coxsackie virus receptor, the coronavirus receptors, and sialic acid and other carbohydrate receptors.

-52-

10. The bifunctional molecule of Claim 5 wherein the
    ligand is selected from the group consisting of
    growth factors, epidermal growth factor,
    interleukins, GM-CSF, G-CSF, M-CSF, EPO, TNF,
5   interferons, and chemokines.

11. The bifunctional molecule of Claim 1 wherein the
    first binding moiety is a single chain antibody
    which binds to a cellular receptor.

12. The bifunctional molecule of Claim 11 wherein the
10 cellular receptor is cell-type specific.

13. The bifunctional molecule of Claim 1 wherein the
    second binding moiety is a polypeptide which has an
    amino acid sequence which is the same or
    substantially the same as an amino acid sequence of
15 at least the viral-binding portion of a native
    extracellular domain of a viral cellular receptor.

14. The bifunctional molecule of Claim 13 wherein the
    second binding moiety has an amino acid sequence
    which is the same as an amino acid sequence of at
20 least the viral-binding portion of a native
    extracellular domain of the viral cellular
    receptor.

15. The bifunctional molecule of Claim 13 wherein the
    native extracellular domain of the viral cellular
25 receptor is on a cell which is subject to infection
    by the viral vector.

-53-

16. The bifunctional molecule of Claim 15 wherein the
    viral vector is selected from the group consisting
    of retroviral vectors, adenoviral vectors,
    parvovirus, coronavirus, orthomyxovirus,
    rhabdovirus, paramyxovirus, picornavirus,
    alphavirus, adenovirus, herpesvirus, poxvirus,
    Norwalk virus, togavirus, flavivirus, reoviruses,
    papovavirus, hepadnavirus, and hepatitis virus.

17. The bifunctional molecule of Claim 15 wherein the
    viral vector is selected from the group consisting
    of a retroviral vector or viral vector which
    comprises a retroviral envelope protein.

18. The bifunctional molecule of Claim 15 wherein the
    viral cellular receptor is epidermal growth factor
    receptor.

19. The bifunctional molecule of Claim 1 further
    comprising a linking moiety.

20. A bifunctional molecule comprising:
    (a) a first binding polypeptide which binds to a
         surface molecule on a target cell, wherein
         said first binding polypeptide is a ligand to
         a cell-type specific cellular receptor;
    (b) a second binding polypeptide which binds to a
         surface molecule on a viral vector, wherein
         the second binding polypeptide is a
         polypeptide which has an amino acid sequence
         which is the same or substantially the same as
         an amino acid sequence of at least the viral-

-54-

binding portion of a native extracellular
domain of a viral cellular receptor.

21.   The bifunctional molecule of Claim 20 wherein the
first cell surface molecule is selected from a
group consisting of carbohydrates, lipids,
polypeptides, and glycosylated polypeptides.

22.   The bifunctional molecule of Claim 20 wherein the
first binding polypeptide and second binding
polypeptide are linked via a peptide bond.

23.   The bifunctional molecule of Claim 22 wherein the
C-terminus of the first binding polypeptide is
linked to the N-terminus of the second binding
polypeptide via a peptide bond.

24.   The bifunctional molecule of Claim 22 wherein the
N-terminus of the first binding polypeptide is
linked to the C-terminus of the second binding
polypeptide via a peptide bond.

25.   The bifunctional molecule of Claim 20 further
comprising a linking moiety.

26.   The bifunctional molecule of Claim 25 wherein the
linking moiety is a polypeptide linker.

27.   The bifunctional molecule of Claim 26 wherein the
C-terminus of the first binding polypeptide is
linked to the N-terminus of the second binding
polypeptide via the polypeptide linker.

28.   The bifunctional molecule of Claim 26 wherein the
       N-terminus of the first binding polypeptide is
       linked to the C-terminus of the second binding
       polypeptide via the polypeptide linker.

5   29.   The bifunctional molecule of Claim 26 wherein the
           polypeptide linker is an immunoglobulin hinge
           region.

    30.   The bifunctional molecule of Claim 28 wherein the
           molecule is expressed from a single nucleic acid
10   molecule.

    31.   A bifunctional molecule for targeting a retroviral
           vector to a cell-type comprising:
           (a)   a receptor-binding fragment of a polypeptide
                  ligand which binds a cellular receptor
15   specific to the cell-type;
           (b)   a viral-binding fragment of a cellular
                  receptor for the retroviral vector.

    32.   The bifunctional molecule of Claim 31 wherein the
           polypeptide ligand is human epidermal growth
20   factor.

    33.   The bifunctional molecule of Claim 31 wherein the
           retroviral vector is avian leukosis virus and the
           cellular receptor for the retroviral vector is Tva.

    34.   The bifunctional molecule of Claim 31 further
25   comprising a hinge region linking the receptor-
           binding fragment to the viral-binding fragment.

-56-

35.   A nucleic acid molecule which encodes a
      bifunctional molecule of Claim 1.

36.   A nucleic acid molecule which encodes a
      bifunctional molecule of Claim 20.

5   37.   The nucleic acid molecule of Claim 36 wherein the
      bifunctional molecule is encoded in a single open
      reading frame.

38.   A nucleic acid molecule which encodes a
      bifunctional molecule of Claim 31.

10   39.   The nucleic acid molecule of Claim 38 wherein the
      bifunctional molecule is encoded in a single open
      reading frame.

40.   A method for preparing a bifunctional molecule
      comprising:

15   (a)   a first binding moiety which binds to a
                 surface
                 molecule on a target cell; and
           (b)   a second binding moiety which binds to a
                 surface molecule on a viral vector, wherein
20   said second binding moiety is an extracellular
                 domain of a viral cellular receptor or a
                 derivative thereof and said bifunctional
                 molecule is not an immunoadhesin;
      wherein the method comprises maintaining a host
25   cell which expresses the bifunctional molecule
      under conditions suitable for expression and
      recovering the bifunctional molecule.

-57-

41.   The method of Claim 40 wherein the first cell
       surface molecule is selected from a group
       consisting of carbohydrates, lipids, polypeptides,
       and glycosylated polypeptides.

5   42.   A method for preparing a bifunctional molecule
            comprising conjugating a first binding moiety which
            binds to a surface protein on a target cell and a
            second binding moiety which binds to a surface
            protein on a viral vector, wherein said second
10   binding moiety is an extracellular domain of a
            viral cellular receptor or a derivative thereof.

     43.   A method for delivering a viral vector to a cell
            comprising contacting the cell with the viral
            vector and a bifunctional molecule comprising:
15   (a)   a first binding moiety which binds to a
                   surface molecule of the target cell; and
            (b)   a second binding moiety which binds to a
                   surface molecule on a viral vector, wherein
                   said second binding moiety is an extracellular
20   domain of a viral cellular receptor or a
                   derivative thereof.

     44.   The method of Claim 43 wherein the first cell
            surface molecule is selected from a group
            consisting of carbohydrates, lipids, polypeptides,
25   and glycosylated polypeptides.

     45.   The method according to Claim 43 wherein the cell
            is contacted with the viral vector *in vitro*.

46. The method according to Claim 43 wherein the cell is contacted with the viral vector *in vivo*.

47. The method according to Claim 43 wherein the viral vector encodes a heterologous protein.

5 48. The method according to Claim 47 wherein the heterologous protein is selected from the group consisting of immunogens, cytokines, tumor necrosis factor, interferons, interleukins, adenosine deaminase, insulin, T-cell receptors, soluble CD4,
10 epidermal growth factor, human growth factor, blood factors, such as Factor VIII, Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL receptor, negative selection markers, anti-angiogenic factors, Fc receptors, plasminogen
15 activators, dopamine, MHC, tumor suppressor genes, monoclonal antibodies or antigen binding fragments thereof, drug resistance genes, ion channels and adrenergic receptors.

49. A method for delivering a viral vector to a cell
20 comprising contacting the cell with the viral vector and a bifunctional molecule comprising:
(a) a first binding polypeptide which binds to a surface molecule on a target cell, wherein said first binding polypeptide is a ligand to
25 a cell-type specific cellular receptor;
(b) a second binding polypeptide which binds to a surface molecule on a viral vector, wherein the second binding polypeptide is a polypeptide which has an amino acid sequence

-59-

which is the same or substantially the same as an amino acid sequence of at least the viral-binding portion of a native extracellular domain of a viral cellular receptor.
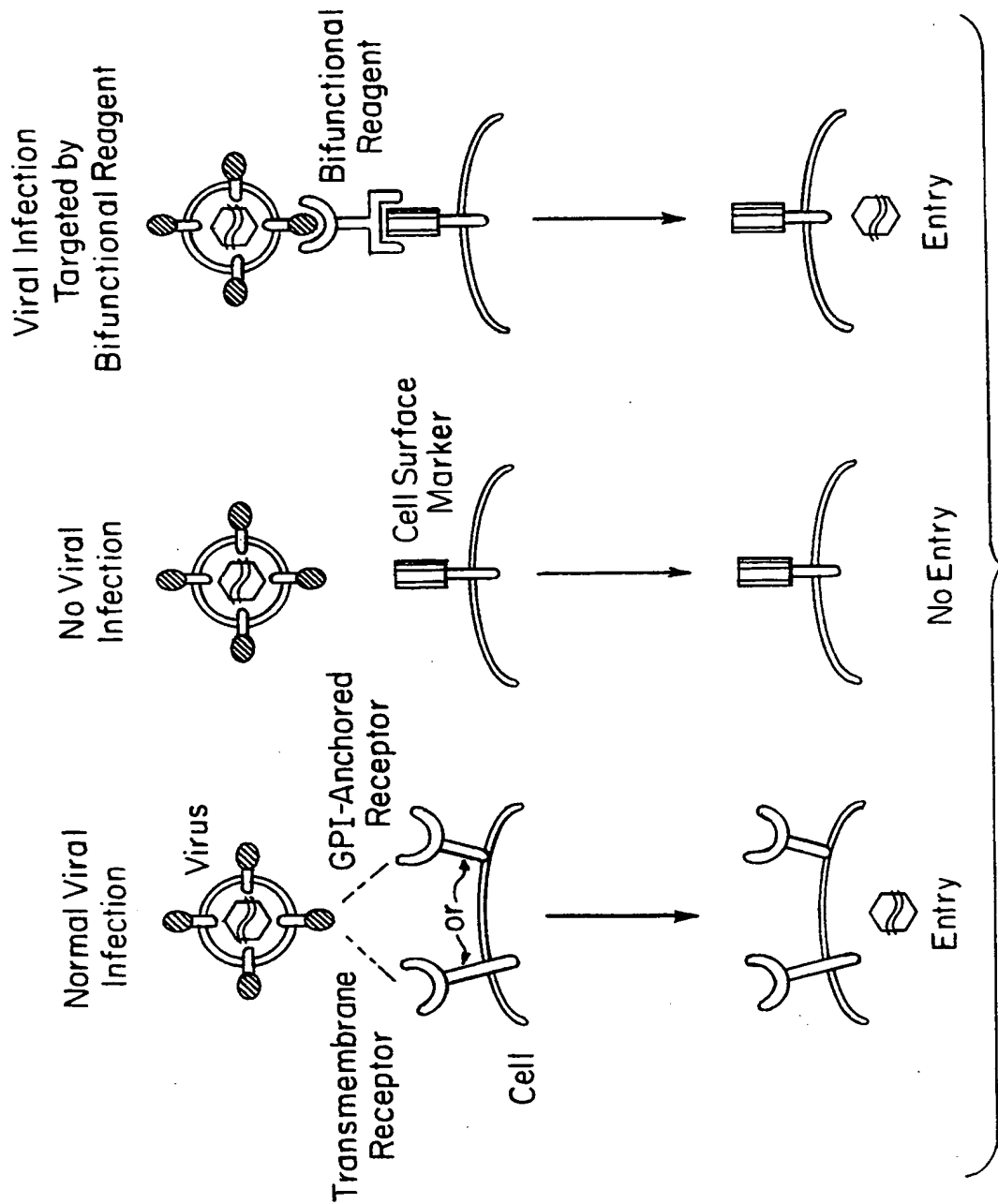
50. The method of Claim 49 wherein the first cell surface molecule is selected from a group consisting of carbohydrates, lipids, polypeptides, and glycosylated polypeptides.

51. A method for delivering a retroviral vector to a cell comprising contacting the cell with the retroviral vector and a bifunctional molecule comprising:

(a) a receptor-binding fragment of a polypeptide ligand which binds a cellular receptor specific to the cell-type;

(b) a viral-binding fragment of a cellular receptor for the retroviral vector.

52. The method according to Claim 51 wherein the cell is contacted with the retroviral vector *in vitro*.

53. The method according to Claim 51 wherein the cell is contacted with the retroviral vector *in vivo*.

54. The method according to Claim 51 wherein the retroviral vector encodes a heterologous protein.

55. The method according to Claim 54 wherein the heterologous protein is selected from the group consisting of immunogens, cytokines, tumor necrosis

factor, interferons, interleukins, adenosine
deaminase, insulin, T-cell receptors, soluble CD4,
epidermal growth factor, human growth factor, blood
factors, such as Factor VIII, Factor IX, cytochrome
5   b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL
receptor, negative selection markers, anti-
angiogenic factors, Fc receptors, plasminogen
activators, dopamine, MHC, tumor suppressor genes,
monoclonal antibodies or antigen binding fragments
10   thereof, drug resistance genes, ion channels and
adrenergic receptors.

56.   The method according to Claim 54 wherein the
polypeptide ligand is human epidermal growth
factor.

15   57.   The method according to Claim 56 wherein the
retroviral vector is avian leukosis virus and the
cellular receptor for the retroviral vector is Tva.

58.   The method according to Claim 57 wherein the
bifunctional molecule further comprising a hinge
20   region linking the receptor-binding fragment to the
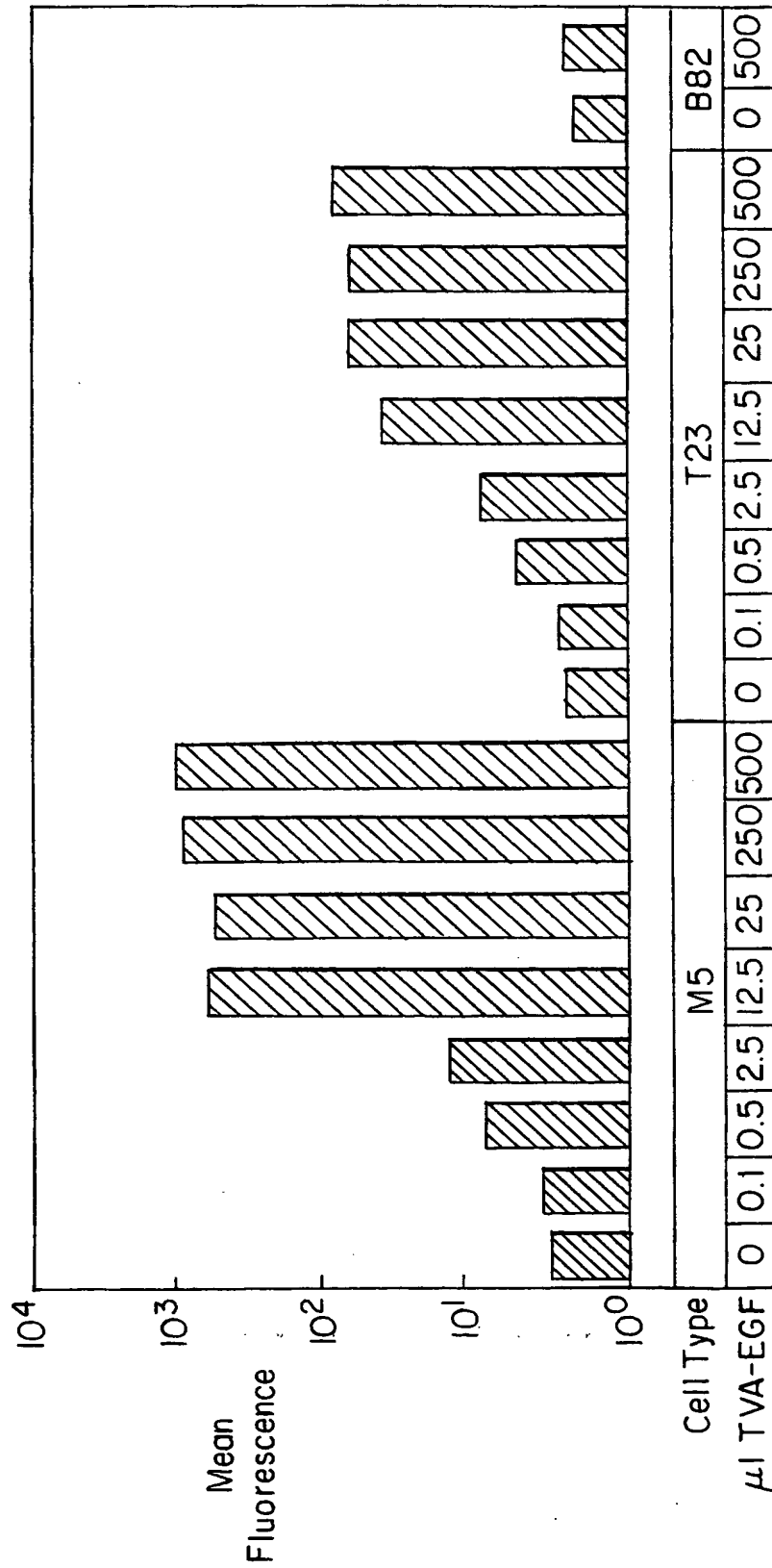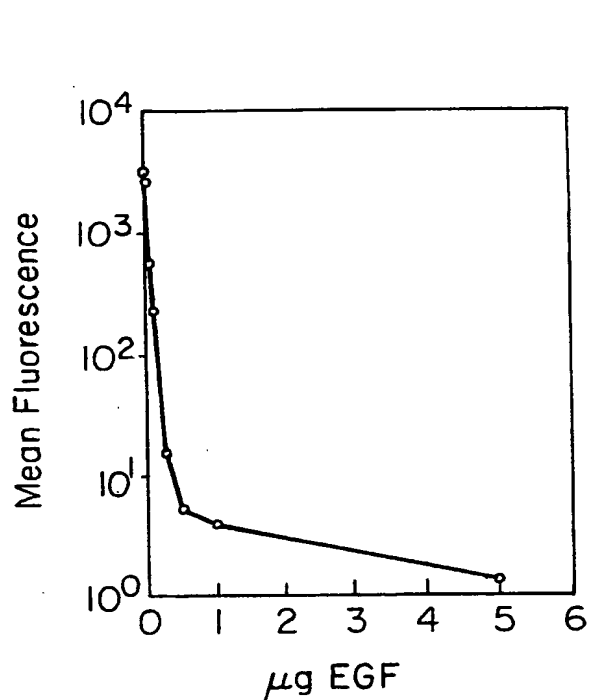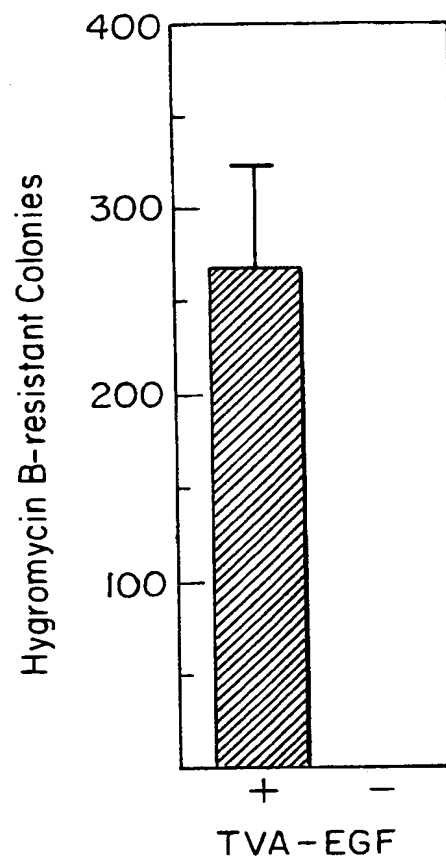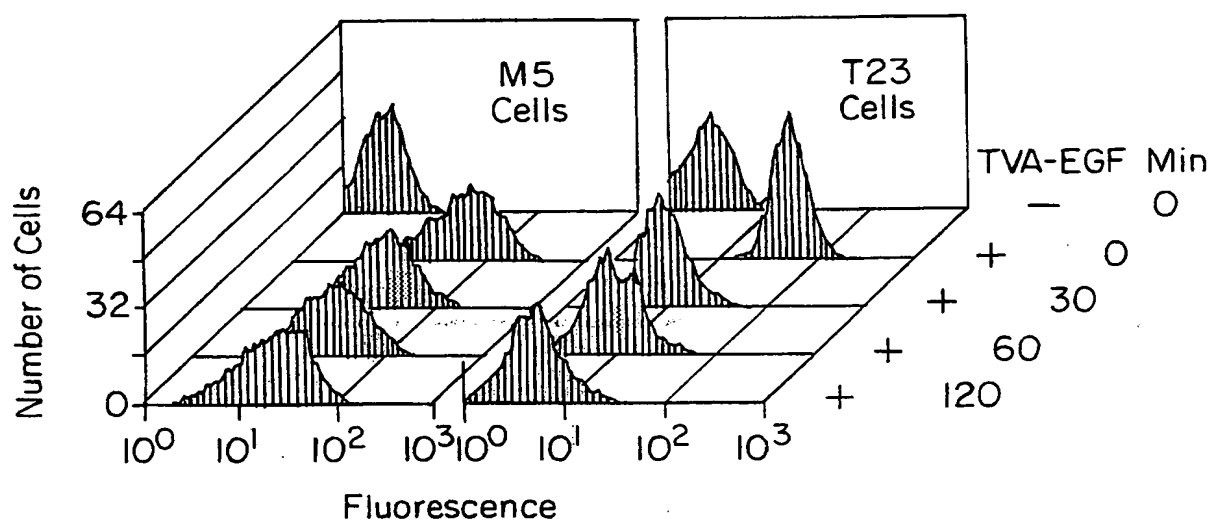viral-binding fragment.

FIG. 1

FIG. 2

FIG. 3

FIG. 5

FIG. 4

FIG. 6

FIG. 7

FIG. 8

Cell Type	M5		
μl TVA-EGF	200		
μg EGF	0	0.5	5
μl RCASA-Neo	150		

(y-axis: G418 Resistant Colonies; 0, 50, 100, 150)

ALV-A
Env

TVA

EGF

EGFR

FIG. 9

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/00 C12N15/62

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 27643 A (TARGETED GENETICS CORP ;PAUL RALPH W (US); OVERELL ROBERT (US)) 8 December 1994 see page 7, line 22 – page 9, line 23 see page 13, line 31 – page 16, line 28 see page 38, line 1 – page 40, line 5 ---	1-58
X	FR 2 649 119 A (CENTRE NAT RECH SCIENT) 4 January 1991 see page 1, line 31 – page 2, line 25 see claims 1-15 ---	1-58
X	WO 93 25682 A (UNIV NEW YORK ;MERUELO DANIEL (US); YOSHIMOTO TAKAYUKI (JP)) 23 December 1993 see page 51, line 27 – page 52, line 15 ---	1-58

-/--

X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 September 1998	06/10/1998

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Seegert, K

Form PCT/ISA/210 (second sheet) (July 1992)

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication.where appropriate. of the relevant passages	Relevant to claim No.
X	WO 93 09221 A (THERA GENE HB) 13 May 1993 see page 1, line 30 - page 2, line 13 ---	1-58
X	ETIENNE-JULAN M ET AL: "THE EFFICIENCY OF CELL TARGETING BY RECOMBINANT RETROVIRUSES DEPENDS ON THE NATURE OF THE RECEPTOR AND THE COMPOSITION OF THE ARTIFICIAL CELL-VIRUS LINKER" JOURNAL OF GENERAL VIROLOGY, vol. 73, December 1992, pages 3251-3255, XP000612121 see abstract -----	1-58

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9427643	A	08-12-1994	AU	7097494 A	20-12-1994
			US	5736387 A	07-04-1998
FR 2649119	A	04-01-1991	NONE		
WO 9325682	A	23-12-1993	AU	4532293 A	04-01-1994
			CA	2137547 A	23-12-1993
			EP	0644936 A	29-03-1995
			JP	7509599 T	26-10-1995
WO 9309221	A	13-05-1993	SE	503225 C	22-04-1996
			CA	2122613 A	13-05-1993
			EP	0614486 A	14-09-1994
			JP	7502026 T	02-03-1995
			SE	9103183 A	01-05-1993
			US	5695991 A	09-12-1997

Form PCT/ISA/210 (patent family annex) (July 1992)